PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Confirmation No. 8055
	)	
Slater, *et al.*	)	Group Art No. 1655
	)	
Serial No.: 10/825,607	)	Examiner: Bin Shen
	)	
Filed: April 16, 2004	)	Docket No: 024730.00015

For: ASSAY METHODS AND MATERIALS

BRIEF ON APPEAL

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Sir:

Appellants submit this Appeal Brief. Please charge any outstanding fee associated with this filing to our Deposit Account No. 19-0733.

STATEMENT OF THE REAL PARTIES IN INTEREST

The real party in interest in this application is Cambrex Bio Science Nottingham, Ltd., the assignee of the application. Cambrex Bio Science Nottingham, Ltd. has changed its name to Lonza Nottingham, Ltd. and is a wholly owned subsidiary of Lonza Group Ltd.

STATEMENT OF RELATED CASES

There are no related cases, including any pending appeals, interferences, or judicial proceedings.

STATUS OF CLAIMS

Claims 1-34 and 44-54 are pending. Claims 1-34 and 44-54 stand finally rejected and are the subject of this appeal. Claims 35-43 have been cancelled. Claims 1, 32, 33, 49, 50, 51, and 54 are the independent claims. The remaining claims are dependent either directly or indirectly on these claims. Appellants appeal the rejection of all of the finally rejected claims. Appendix 1 presents a copy of the claims involved in this appeal.

STATUS OF AMENDMENTS

No amendments to the claims have been made subsequent to final rejection. Appendix 1 provides a copy of the claims as they were pending at the time of the final rejection.[1] A Jurisdictional Statement also is appended as Appendix 4.

SUMMARY OF CLAIMED SUB JECT MATTER

The independent claims, *i.e.,* claims 1, 32, 33, 49, 50, 51, and 54, are directed respectively to a method for detecting the presence of contaminating mycoplasma in a test sample (claim 1); to a process for treating a cell culture to remove mycoplasma contamination (claim 32); and to various methods for detecting the presence of contaminating mycoplasma in a test sample (claims 33, 49, 50, 51 and 54).

Annotated Summaries for Independent Claims 1, 32, 33. 49, 50, 51 and 54:

Independent Claim 1 embraces a method for detecting the presence of

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1 Claim 52 has a minor informality that needs correction in any proceedings following this appeal. Four lines from the end of claim 52, "ration" should be "ratio."

contaminating mycoplasma (page 2, lines 2-5)[2] in a test sample in which a test sample (page 6, lines. 28-29; page 14, lines 17-19; and page 15, line 1 to page 16, line 30) is provided and then the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof is detected and/or measured in the test sample (page 7, line 26 to page 8, line 7; page 12, lines 22-31; page 13, lines 5-6; Table 2, page 13; page 21, lines 14-19), the measurement of that activity being indicative of the presence of contaminating mycoplasma (page 7, lines 5-8; page 21, lines 1-3). On the basis of that detection and/or measurement of activity one identifies the test sample as contaminated with mycoplasma (page 7, lines 9-10; page 21, lines 1-7; Figures and 2).

Independent Claim 32 embraces a process for treating a cell culture to remove mycoplasma (page 2, lines 2-5) contamination which involves treating a mycoplasma contaminated cell culture with an agent to remove and/or destroy mycoplasma (page 18, lines 3-9; page 18, line 11 to page 19, line 12; page 22, line 16 to page 23, line 9 and Figure 3); and then subsequently testing a sample from the culture for mycoplasma contamination using the method of **Independent**

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2 The page references throughout the brief are to the specification as originally filed.

Claim 1 or dependent Claim 2; and if necessary, repeating the process of treating one or more times until mycoplasma contamination is not detected in a sample (page 18, lines 6-9; Example 2 and Figure 3).

Independent Claim 33 embraces a method of detecting the presence of mycoplasma (page 2, lines 2-5) in a test sample (page 6, lines. 28-29; page 14, lines 17-19; and page 15, line 1 to page 16, line 30) in which ATP is detected or measured in a test sample without adding an exogenous reagent (e.g., substrates for kinase activity) to convert ADP to ATP, and the ATP is detected or measured in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement (page 17, lines 6-9; page 20, lines 26-30; page 24, line 11 to page 25, line 18). A measurement of ATP and/or light output also is obtained from a corresponding control sample (page 17, lines 10-11 and lines 20-24; page 20, lines 26-30; and by determining the ATP and/or light output measurement ratio as (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample) (page 17, line 12; page 21, lines 1-7; page 34, line 39 to page 35, line 31), one identifies whether the test sample is contaminated with mycoplasma by determining whether the ratio of (ATP and/or light output measurement from the corresponding control

sample)/(ATP and/or light measurement from the test sample) is greater than one (page 17, lines 13-14; page 21, lines 1-7).

Independent Claim 49 relates to a method of detecting the presence of contaminating mycoplasma (page 2, lines 2-5) in a test sample (page 6, lines 28-29; page 14, lines 17-19; and page 15, line 1 to page 16, line 30) where the test sample is treated under a condition sufficient to lyse contaminating mycoplasma in the sample but insufficient to lyse bacterial cells (page 10, lines 12 to page 11, line 5; page 29, line 15 to page 32, line 13) and then the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof is then detected and/or measured in the test sample, the activity being indicative of the presence of contaminating mycoplasma (page 7, line 26 to page 8, line 7; page 12, lines 22-31; page 13, lines 5-6; Table 2, page 13; page 21, lines 14-19; page 30, lines 4-8 and Figure 10; page 34, line 34 to page 35, line 5) The test sample is identified as being contaminated with mycoplasma on the basis of detection and/or measurement of that activity (page 7, lines 9-10; page 21, lines 1-7; Figures 2 and 10).

Independent Claim 50 also embraces a method of detecting the presence of mycoplasma (page 2, lines 2-5) in a test sample (page 6, lines. 28-29; page 14,

lines 17-19; and page 15, line 1 to page 16, line 30) in which the test sample is treated under a condition sufficient to lyse contaminating mycoplasma but insufficient to lyse bacterial cells (page 10, line 12 to page 11, line 5; page 29, line 15 to page 32, line 13) and then without adding an exogenous reagent (e.g., substrates for kinase activity) ADP is converted to ATP, and ATP is detected or measured in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement (page 17, lines 6-9; page 20, lines 26-30; page 24, line 11 to page 25, line 18). An ATP and/or light output measurement also is obtained from a corresponding control sample (page 17, lines 10-11 and lines 20-24; page 20, lines 26-30) and the ATP and/or light output measurement ratio as (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample) is determined (page 17, line 12; page 21, lines 1-7; page 34, line 39 to page 35, line 31). A test sample is identified as contaminated with mycoplasma in the event that the ratio of (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample) is greater than one (page 35, line 31).

Independent Claim 51 relates to a method of detecting the presence of

contaminating mycoplasma (page 2, lines 2-5) in a test sample (page 6, lines. 28-29; page 14, lines 17-19; and page 15, line 1 to page 16, line 30) in which the test sample is passed through a filter which retains bacterial cells (page 21, lines 21-24; page 26, lines 14-23; page 31, lines 14-15; page 32, lines 8-13) and then the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof is detected and/or measured in the test sample (page 7, line 26 to page 8, line 7; page 12, lines 22-31; page 13, lines 5-6; Table 2, page 13; page 21, lines 14-19), with the measured activity being indicative of the presence of contaminating mycoplasma (page 7, lines 5-8; page 21, lines 1-3). The test sample is identified as contaminated with mycoplasma on the basis of the detection and/or measurement of that activity (page 7, lines 9-10; page 21, lines 1-7; Figures and 2).

Independent Claim 54 also embraces a method of detecting the presence of mycoplasma (page 2, lines 2-5) in a test sample (page 6, lines. 28-29; page 14, lines 17-19; and page 15, line 1 to page 16, line 30) in which the test sample is passed through a filter which retains bacterial cells (page 21, lines 21-24; page 26, lines 14-23; page 31, lines 14-15; page 32, lines 8-13) and then without adding an exogenous reagent (e.g., substrates for kinase activity) ADP is converted to ATP

which is detected or measured in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement (page 17, lines 6-9; page 20, lines 26-30; page 24, line 11 to page 25, line 18). An ATP and/or light output measurement is also obtained from a corresponding control sample (page 17, lines 10-11 and lines 20-24; page 20, lines 26-30) and the ATP and/or light output measurement ratio as (ATP and/or light output measurement from the corresponding control sample) / (ATP and/or light measurement from the tests sample) is determined (page 17, line 12; page 21, lines 1-7; page 34, line 39 to page 35, line 31). The test sample is identified as contaminated with mycoplasma in the event that the ratio of (ATP and/or light output measurement from the corresponding control sample) / (ATP and/or light measurement from the test sample) is greater than one (page 35, line 31).

GROUNDS OF REJECTION TO BE REVIEWED

(1) Whether claims 1-5, 8-24, 33 and 44 are non-enabled for test samples with bacteria and certain eukaryotic microbes growth.

(2) Whether claims 1, 3, 4, 10, 13, 14 and 44 are anticipated by **Kahane** (FEMS Microbiology Letters, 1978; 3:143-145).

(3) Whether claims 1-34 and 44-54, *i.e.,* all pending claims under

examination, would have been obvious under 35 U.S.C. 103(a) over the combined teachings from **Kahane** in view of **Ito** (Analytical Sciences 2003;19:105-109).

ARGUMENT

THE ENABLEMENT REJECTION

The Examiner concedes that the specification is enabling for detecting the presence of mycoplasma contamination in mammalian cell cultures, but finally rejects claims that (1) are not focused specifically on the testing of a cell culture, (2) are not directed to a cell-free sample, or (3) are not directed to a sample where the claim expressly requires that the sample be treated in a way specifically aimed at removing or leaving intact bacterial cells.[3]

In making this rejection, the Examiner contends that the specification "does not reasonably provide enablement for any test samples with bacteria and certain eukaryotic microbes growth (such as fungi, see **Ingram-Smith** et al., Trends in Microbiology, 2006:14(6):249-253)."

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3 For convenience these are claims 1-5, 8-24, 33 and 44.

Ignoring that the cited **Ingram-Smith** article is a post-filing date reference and thus should not be considered as part of an enablement challenge (See *In re Hogan,* 559 F.2d 595, 194 USPQ 527 (CCPA 1977) and *United States Steel Corp. v. Phillips Petroleum Co.,* 865 F.2d 1247, 9 USPQ2d 1461 (Fed. Cir. 1989)) and even further ignoring the admission by the reference that "these enzymatic activities have not yet been demonstrated in eukaryotic microbes" (see page 252, right hand column, lines 18-19), applicants fail to see how the potential interference from other microbes impacts the enablement of the pending claims.

The rejection appears to be based on the assumption that the presence of bacteria or eukaryotic microbes in a sample would render the claimed method inoperative. The Examiner has not provided any evidence that the methods embraced by the rejected claims will not identify the existence of contaminating mycoplasma. The assumption is based solely on the Examiner's speculation.

Indeed, the evidence in the specification is to the contrary. The specification demonstrates that the method can be practiced in the presence of bacteria and in that circumstance can successfully identify *mycoplasma* contamination. See especially Example 7, pages 29-32, including Figures 10 and 11. Furthermore, the application on several occasions teaches techniques for analyzing samples

containing bacteria, *inter alia,* see page 10, line 12 to page 13, line 5. To the extent there are other microbes that could potentially complicate the assay method; one skilled in the art would recognize that the techniques for accommodating them would be the same as those used for bacteria. Claims are not required to include limitations that those skilled in the art would consider apparent. *In re Skrivan,* 427 F.2d 801, 166 USPQ 85 (CCPA 1970).

Further, even if the presence of another microbe in a sample caused a positive result in the test (presumably because the other microbe contained an active enzyme with a similar activity to the *mycoplasma* enzymes sought to be detected), the potential generation of such a possible "false positive" result is not indicative of a lack of enablement of the claimed invention. False positive results are a potential outcome in many assays and are not indicative that an assay is either non-enabled or inoperative. Such results are a fact of life and can be dealt with a variety of ways, some of which are described in the specification.[4] In any event,

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4 For bacteria in particular, in addition to the disclosure of using bacterial filters or selective lysis, the specification also notes that bacterial contamination can be identified independently by the presence of turbid growth or by using phase

there is no evidence of record showing that any *mycoplasma* present in the tested sample would not similarly be detected, *i.e.,* that the presence of other microbes would prevent the detection of *mycoplasma*, if present. The only potential drawback is that in some particular test, in the absence of further investigation, there may be some uncertainty about the cause for the result. That complication, however, does not amount to a lack of enablement.

The disclosure needed to comply with the enablement requirement of 35 USC 112 varies with the scope of the claimed invention. *CFMT, Inc. v. YieldUP International Corp.*, 349 F.3d 1333, 1338, 68 USPQ2d 1940 (Fed. Cir. 2003). Here, the methods embraced by the rejected claims do not require a foolproof assay and thus the claims need not be supported by a specification that requires a foolproof result. The rejected claims are enabled.

THE ANTICIPATION REJECTION

For purposes of this rejection, appellants acknowledge that claim 1 can be considered representative of the rejected claims.

A claim is anticipated only if each and every element set forth in the claim is

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contrast microscopy (page 11, lines 7-10).

found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987); *PPG Industriies, Inc. v. Guardian Industries Corp., 75 F.3d 1558, 1566, 37 USPQ2d 1618, 1624 (Fed. Cir. 1996); Atofina v. Great Lakes Chemical Corp., 441 F.3d 991, 999, 78 USPQ2d 1417 (Fed. Cir. 2006).* The disclosure of the claimed invention in the reference must be so clear and unequivocal that a skilled worker is not left to pick and choose among various options. *In re Arkley,* 455 F.2d 586, 587, 172 USPQ 524 (CCPA 1972). If the basis of the anticipation is inherency, then the extrinsic evidence must make it clear that the missing disclosure is necessarily and invariably present; inherency is NOT established by probabilities or possibilities, *Crown Operations International, Ltd. v. Solutia Inc.*, 289 F.3d 1367, 1377 (Fed. Cir. 2002); *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949 (Fed. Cir. 1999).

As recited, claim 1 focuses on a method for detecting the presence of "contaminating mycoplasma" in a test sample (see also page 1, lines 8-10 of the specification). The recitation of "contaminating mycoplasma" in the preamble of claim 1 is re-emphasized in the body of the claim where the detection and/or measurement of enzyme activity is "indicative of the presence of contaminating

mycoplasma" and a sample is identified as "contaminated" based on that detection and/or measurement.

Appellants contend that by using the terms "contaminating," "contaminated," or "contamination" in the claims in connection with the detection and/or measurement of mycoplasma, the claims, as properly construed, embrace only those methods where (1) it is understood that the "test sample" is a sample that does not intentionally contain mycoplasma, (2) it is understood that a "test sample" is a sample for which it is not known whether it contains mycoplasma contamination and (3) it is understood that to the extent any mycoplasma is present in the "test sample" it is likely to be present only in a small "contaminating" amount.

The cited **Kahane** reference fails to anticipate these claims because **Kahane** does not provide a "test sample" within the meaning of claim 1, *i.e.,* a sample that must be tested for acetate kinase/carbamate kinase activity to determine whether it contains any "contaminating mycoplasma." **Kahane** already knew that the isolated material tested during the reported research contained acetate kinase, because the isolated mycoplasma was cultivated specifically for that purpose.

Kahane is an academic article relating to the identification and biochemical

characterization of acetate kinase in pure mycoplasma cultures. In particular, **Kahane** presents the results of a study aimed at determining whether (*i.e.*, assessing the hypothesis that) acetate kinase (AK) acts as a supplier of ATP in mycoplasma as it does in anaerobic bacteria. In this regard, **Kahane** describes the cultivation of mycoplasma cells of two species, *A. laidlawii* and *M. hominis* (18-22 hours at 37 °C), harvesting the cells from that cultivation, and the isolation and analysis of the acetate kinase recovered from the harvested cells. **Kahane** thus produces an isolated preparation of acetate kinase from pure mycoplasma cell preparations of both *A. laidlawii* and *M. hominis* and then measures the enzymatic activity of the isolated material. **Kahane** does not suggest, nor disclose analyzing a sample not known to contain mycoplasma for acetate kinase activity.

Claims 1 and the related dependent claims 3, 4, 10, 13, 14 and 44 are not anticipated by **Kahane**.

THE OBVIOUSNESS REJECTION

Kahane establishes the presence of acetate kinase in mycoplasma – but that is all **Kahane** does. Indeed, **Kahane** deliberately cultivated pure cultures of mycoplasma and isolated a homogeneous preparation of acetate kinase for the very purpose of investigating the physiological role played by acetate kinase in

mycoplasmas. Working with pure cultures of mycoplasmas and with homogeneous preparations of acetate kinase, however, is a far cry from developing an assay for determining whether a particular "test sample" that is intended to be free of mycoplasma is nonetheless "contaminated" with a mycoplasma.

In framing the obviousness rejection, the Examiner combines **Kahane** with **Ito**. **Ito** relates to a bioluminescent approach for simultaneously assessing acetate kinase and pyruvate phosphate dikinase activities. In particular, **Ito** used acetate kinase activity as one of the enzymatic reporters in a tandem immunoassay for assaying insulin and C-peptide in a single sample. **Ito** used pyruvate phosphate dikinase from *Microbisora rosea subsp. Aerata* and acetate kinase from *B. stearothermophilus*. Nothing in **Ito** links the acetate kinase to mycoplasmas.

Apparently, it is the Examiner's position that a skilled worker knowing that mycoplasma contamination is a potential problem would have understood (1) from **Kahane** that mycoplasma could be detected by assaying for acetate kinase activity, (2) that the **Ito** assay could be used for that purpose and (3) that running a control assay was routine and within the skill of the art. Appellants submit that the rejection improperly uses hindsight to select teachings from the prior art and to evaluate how those teachings might have been used in combination by a skilled

worker.

As the Federal Circuit cautioned in *In re Dembiczak*, 175 F.3d 994, 50 U.S.P.Q.2d 1614 (Fed. Cir. 1999), "[m]easuring a claimed invention against the standard established by section 103 requires the oft-difficult but critical step of casting the mind back to the time of invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field." The fact finder must avoid the "insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against the teacher." *In re Fine*, 837 F.2d 1071, 1075, 5 U.S.P.Q.2d 1596, 1600 (Fed. Cir. 1988). Indeed, in its recent KSR decision (*KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727 (2007)), the Supreme Court also cautioned against using hindsight in the patentability analysis stating that "[a] factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning."

Kahane was published in 1978, over twenty-five (25) years prior to applicant's invention. During that time, mycoplasma contamination had long been

recognized as a significant though continuing problem. (page 3, lines 17-30[5]; Rottem and Barile (1993) and McGarrity and Kotani (1985) – identified in Appendix 2).

Attempts at solving the problem have included *in vitro* culturing (page 4, lines 1-19); DNA analysis using fluorochrome (page 4, lines 21-31); PCR analysis (page 5, lines 1-16); Life Technologies' MYCOTECT kit, which examines the activity of adenosine phosphorylase (page 5, lines 18-30) and immunoassays (page 6, lines 1-7). As a general rule, these assays are time and labor intensive and are complicated to perform. Appellants submit that existence and availability of **Kahane**'s teachings throughout the development of these competing technologies, without even a passing reference to the use of acetate kinase/carbamate kinase activity as a technique for gauging mycoplasma contamination, is persuasive evidence of the non-obviousness of appellants' invention.

Appellants submit that this history underscores the fact that a skilled worker would never have considered **Kahane**'s teachings in the context of developing a method for detecting small "contaminating" amounts of mycoplasma in a "test sample." Only with the hindsight knowledge of appellants' invention, would a

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5 See footnote 2.

skilled worker (or anyone for that matter) have any basis to identify **Kahane** or any reason for consulting **Kahane**'s teachings as potentially relevant to the present invention. **Kahane**'s selection as a reference by the Examiner represents a classic case of the improper use of hindsight. For that reason, the rejection fails to present a *prima facie* case of obviousness.

Moreover, even if a skilled worker would have found it obvious to implement **Ito**'s bioluminescent approach for measuring acetate kinase activity, in place of **Kahane**'s relatively crude enzyme-coupled detection system, that recognition does not put the present invention in the hands of a skilled worker. In that case, **Ito**'s bioluminescent assay simply serves as an alternative way for analyzing the physiological role played by acetate kinase in mycoplasmas. A skilled worker would never have considered **Kahane**'s research in the context of developing a method for detecting small, "contaminating" amounts of mycoplasma in a sample.

Appellants also question whether, in the absence of impermissible hindsight, a skilled worker would ever have considered **Ito** in combination with **Kahane**. As with **Kahane**, **Ito** has nothing to do with assessing the presence of contaminating mycoplasma in a sample. Nothing links these separate, disparate references

besides the pending application and the rejected claims. Nowhere in the rejection is there any explanation of why a skilled worker would have been motivated, as a consequence of these references, to develop an assay designed to assess mycoplasma contamination, or why a skilled worker would have selected these references in that endeavor.

The Examiner has therefore failed to present a *prima facie* case that all of the pending claims are obvious.

Lack of a proper *prima facie* case of obviousness is especially evident when considering the rejection in the context of various other claims directed to preferred aspects of the invention.

THE "CONTROL CLAIMS"

Claims 2, 7-9, 17-24, 33, 50, 52 and 54, in one fashion or another all require that the assay be run with a parallel "control sample." Each of these "control claims" requires that information obtained from detecting/measuring the acetate kinase/carbamate kinase activity in a "control sample" be compared with the activity detected/measured in the test sample. A particular subset of these claims are claims 19-24 which further require that the control sample have been shown to be free of mycoplasma by a separate method.

To present a sufficient *prima facie* case that these "control claims" would have been obvious to an ordinary skilled worker, at a minimum, the Examiner must explain why a skilled worker presented with **Kahane** and **Ito** would want or need to run a control sample and would want or need to compare the results of that control sample with a second measurement. The Examiner, however, makes no attempt to explain how the cited references would have provided motivation for a skilled worker to analyze a control sample in tandem with a test sample. Instead, the Examiner simply contends that use of a control is "merely a matter of judicious selection and routine optimization." That off-hand remark does not sustain the Examiner's burden of presenting a *prima facie* case of obviousness.

Pointedly, nothing in **Kahane** suggests any need or discloses any benefit from performing a control, particularly a control which has been shown to be free of mycoplasma by a separate method. **Kahane** did not need a control because **Kahane** purposefully cultivated mycoplasmas and purposefully analyzed the acetate kinase isolated from the mycoplasma cultures in order to assess its level of activity and its manner of action. **Kahane**'s investigation was targeted specifically to the study of the role played by acetate kinase in mycoplasmas; it was not designed to assess the possible presence of mycoplasma in a sample which was

intended to be free of mycoplasma. Nor is there any teaching in the secondary reference, **Ito** to cure this glaring deficiency of **Kahane**. On that basis, the rejection of the "control claims," especially claims 19-24, for obviousness must be withdrawn.

THE "SPECIFIC TEST SAMPLE CLAIMS"

Claims 25-31 and 45-48 in one fashion or another all require that the "test sample" constitute a very specific material, and in particular a material in which the presence of mycoplasma would be an undesired characteristic, (*e.g.,* cultures of mammalian or plant cells). The Examiner has not even proffered an explanation of why either of the cited references would have motivated a skilled worker to run the acetate kinase assays that they respectively describe in the context of the specific test samples embraced by these claims.

Again, **Kahane** analyzed the disclosed preparations for acetate kinase activity only because those preparations were intentionally derived from pure cultures of mycoplasma that had been deliberately cultivated for isolating acetate kinase. **Kahane** provides no motivation to use its assay on any sample that is of unknown composition, *i.e.,* that is not already known to contain acetate kinase. If a sample was not known to contain mycoplasmal acetate kinase, why would **Kahane**

have had any interest in analyzing it? **Ito** also deliberately chose acetate kinase as one of two reporter enzymes for the disclosed immunoassay. Because both **Kahane** and **Ito** intentionally introduced acetate kinase into the materials each intended to assay, it is not surprising that each sought to measure or detect acetate kinase in samples of those preparations. There is not a single teaching in either reference, however, that would have motivated a skilled worker to perform an acetate kinase assay on any of the specific "test samples" or "control samples" embraced by the present claims. On that basis, the rejection of the above identified specific test sample claims for obviousness must be withdrawn.

THE "ABSENCE OF EXOGENOUS REAGENT CLAIMS"

Claims 33, 50 and 54 each requires that the process (assay) be conducted "without adding an exogenous reagent to convert ADP to ATP." Example 5 describes this technique (page 24, line 11 to page 25, line 17). As with the "specific test sample claims," the Examiner has not proffered an explanation of why either of the cited references would have motivated a skilled worker to run the acetate kinase assays in the manner embraced by these latter "absence of endogenous reagent claims;" nor why a skilled worker would have had a reasonable expectation of success in conducting the assay in such a manner.

To present a sufficient *prima facie* case that these claims would have been obvious to an ordinary skilled worker, at a minimum, the Examiner must explain why a skilled worker presented with **Kahane** and **Ito** would have had a reasonable expectation of successfully performing the assay without the addition of exogenous reagents to convert ADP to ATP. As there does not appear to be any disclosure relevant to this issue in either of the cited references, the rejection of the above-identified "absence of exogenous reagent claims" for obviousness must be withdrawn.

THE "BACTERIAL FILTER CLAIMS"

Claims 34, 51-54 each requires a step in the process (assay) of "passing the test sample through a filter which retains bacterial cells." As with the "specific test sample claims," the Examiner has not proffered an explanation of why either of the cited references would have motivated a skilled worker to run the acetate kinase assays in the manner embraced by these latter claims. To present a sufficient *prima facie* case that these claims would have been obvious to an ordinary skilled worker, at a minimum, the Examiner must explain why a skilled worker presented with **Kahane** and **Ito** would have been motivated to perform this step of bacterial filtration. Neither reference contains any disclosure that would even remotely

suggest a reason for, or the benefit of, performing such a filtration step in connection with an acetate kinase assay. As a result, the rejection of the "bacterial filter claims" for obviousness must be withdrawn.

THE "SELECTIVE BACTERIAL LYSIS CLAIMS"

Claims 6, 7, 49 and 50 each requires a step in the process (assay) of subjecting the test sample to a lysis treatment that is "not capable of lysing bacterial cells." As with the "specific test sample claims," the Examiner has not proffered an explanation of why either of the cited references would have motivated a skilled worker to run the acetate kinase assays in the manner embraced by these "selective bacterial lysis claims." To present a sufficient *prima facie* case that these claims would have been obvious to an ordinary skilled worker, at a minimum, the Examiner must explain why a skilled worker presented with **Kahane** and **Ito** would have been motivated to perform this step of selective bacterial lysis. Neither reference contains any disclosure that would even remotely suggest a reason for, or the benefit of, performing such a step in connection with an acetate kinase assay. As a result, the rejection of the above-identified "selective bacterial lysis claims" for obviousness must be withdrawn.

THE "CELL CULTURE TREATMENT CLAIM"

Claim 33 recites a process for treating a cell culture to remove mycoplasma contamination. As with the "specific test sample claims," the Examiner has not proffered an explanation of why either of the cited references would have motivated a skilled worker to use the acetate kinase assays in the manner embraced by the cell culture treatment of this claim. To present a sufficient *prima facie* case that this claim would have been obvious to an ordinary skilled worker, at a minimum, the Examiner must explain why a skilled worker presented with **Kahane** and **Ito** would have been motivated to perform the required treatment step. Neither reference contains any disclosure that would even remotely suggest a reason for, or the benefit of, performing such a step in connection with an acetate kinase assay. Indeed, treating the sample to eradicate the mycoplasma would have been antithetical to the very purpose of the **Kahane** research studying mycoplasma enzymes. As a result, the rejection of the "cell culture treatment claim" for obviousness must be withdrawn.

CONCLUSION

When hindsight is removed from the analysis, as it must be, one is left with

prior art teachings that do not disclose, or even remotely suggest that the claimed subject matter could be successfully produced. For the reasons given above, all rejections of the pending claims under 35 U.S.C. §§102(b), 103(a) and 112, ¶ 1, are improper. The Board of Patent Appeals and Interferences should reverse these rejections. That reversal is respectfully requested.

Respectfully submitted,

/Joseph M. Skerpon/

Date: March 6, 2009 By: _____

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APPENDIX 1. APPEALED CLAIMS

Claim 1. A method of detecting the presence of contaminating mycoplasma in a test sample comprising:

(i) providing a test sample;

(ii) detecting and/or measuring the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof in the test sample, and said activity being indicative of the presence of contaminating mycoplasma; and

(iii) identifying the test sample as contaminated with mycoplasma on the basis of detection and/or measurement of said activity in step (ii).

Claim 2. The method of claim 1 further comprising the following steps performed after step (ii) but before step (iii):

(iia) obtaining enzyme activity information of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture

thereof, detected and/or measured in a corresponding control sample; and

(iib) comparing the activity detected and/or measured in the test sample in step (ii) of claim 1 with the activity detected and/or measured in the control sample in step (iia);

wherein the test sample is identified as contaminated with mycoplasma in step (iii) if the activity detected and/or measured in the test sample in step (ii) is greater than the activity detected and/or measured in the control sample in step (iia), that is, the ratio of the activity detected and/or measured in the test sample in step (ii) to the activity detected and/or measured in the control sample in step (iia) is greater than one.

Claim 3. The method of claim 1 or 2 wherein detecting and/or measuring the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture thereof in the test sample in step (ii) and/or obtaining enzyme activity information of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture thereof in a corresponding control sample in step (iia) comprises detecting and/or measuring

the appearance and/or disappearance of one or more of the substrates and/or one or more of the products of the following reactions:

(*Ri*) acetyl phosphate + ADP $\overset{\text{acetate kinase}}{\longleftrightarrow}$ acetate + ATP

(*Rii*) carbamoyl phosphate + ADP $\overset{\text{carbamate kinase}}{\longleftrightarrow}$ ammonia + carbonate + ATP.

Claim 4. The method of claim 3 further comprising the step of releasing mycoplasma cellular contents into the sample by treatment of the test sample with a mycoplasma lysis agent that is performed after step (i) but before step (ii).

Claim 5. The method of claim 4 wherein the lysis agent is a detergent.

Claim 6. The method of claim 5 wherein the detergent lysis treatment is not capable of lysing bacterial cells.

Claim 7. The method of claim 6 wherein the corresponding control sample is

the same as the test sample prior to mycoplasma lysis treatment.

Claim 8. The method of claim 2 wherein the corresponding control sample is the same as the test sample but the step of obtaining detection/measurement for the test sample activity information is carried out after a time interval following the step of obtaining detection/measurement information for the control sample.

Claim 9. The method of claim 8 wherein the time interval is at least approximately 30 minutes.

Claim 10. The method of claim 1 or 2 wherein the detecting and/or measuring step comprises detecting and/or measuring ATP.

Claim 11. The method of claim 10 wherein the ATP is detected and/or measured by a light-emitting reaction.

Claim 12. The method of claim 11 where the light emitting reaction is a

bioluminescent reaction.

Claim 13. The method of claim 10 wherein ADP is added to the test sample prior to the detecting and/or measuring step (ii).

Claim 14. The method of claim 1 or 2 wherein a mycoplasma substrate (MS) reagent is added to the test sample prior to the detecting and/or measuring step (ii).

Claim 15. The method of claim 44 wherein the precursor of acetyl phosphate is acetyl-CoA.

Claim 16. The method of claim 44 wherein the precursor of carbamoyl phosphate is selected from the group consisting of citrulline, ammonia and a mixture thereof.

Claim 17. The method of claim 13 wherein the control sample is all or an aliquot of the test sample to which a mycoplasma reagent has not been added.

Claim 18. The method of claim 14 wherein the control sample is all or an aliquot of the test sample to which a mycoplasma reagent has not been added.

Claim 19. The method of claim 2 wherein the control sample has been shown to be free from mycoplasma by a separate method.

Claim 20. The method of claim 10 wherein the control sample has been shown to be free from mycoplasma by a separate method.

Claim 21. The method of claim 14 wherein the control sample has been shown to be free from mycoplasma by a separate method.

Claim 22. The method of claim 19 wherein the control sample has been shown to be free from mycoplasma by one or more of PCR testing, DNA fluorescence staining, or mycoplasma culture method.

Claim 23. The method of claim 20 wherein the control sample has been shown

to be free from mycoplasma by one or more of PCR testing, DNA fluorescence staining, or mycoplasma culture method.

Claim 24. The method of claim 21 wherein the control sample has been shown to be free from mycoplasma by one or more of PCR testing, DNA fluorescence staining, or mycoplasma culture method.

Claim 25. The method of claim 1 or 2 wherein the test sample and/or control sample is a cell-culture sample.

Claim 26. The method of claim 25 wherein cells in the cell-culture sample are mammalian cells.

Claim 27. The method of claim 26 wherein the mammalian cells in the cell-culture sample grow in suspension.

Claim 28. The method of claim 25 where the cell culture is a culture of plant cells.

Claim 29. The method of claim 25 where the cell culture sample is a sample which is derived from a cell culture but is itself substantially free of cellular material.

Claim 30. The method of claim 1 or 2 wherein the test sample and/or control sample consists of a cell-free reagent.

Claim 31. The method of claim 30 where the cell-free reagent is trypsin.

Claim 32. A process for treating a cell culture to remove mycoplasma contamination comprising: treating a mycoplasma contaminated cell culture with an agent to remove and/or destroy mycoplasma; and subsequently testing a sample from the culture for mycoplasma contamination using the method of claim 1 or 2; if necessary, repeating the process of treating one or more times until mycoplasma contamination is not detected in a sample.

Claim 33. A method of detecting the presence of mycoplasma in a test sample,

comprising the following steps:

(i) providing a test sample;

(ii) without adding an exogenous reagent (e.g. substrates for kinase activity) to convert ADP to ATP, detecting or measuring ATP in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement;

(iii) obtaining an ATP and/or light output measurement from a corresponding control sample;

(iv) determining the ATP and/or light output measurement ratio as (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample); and

(v) identifying the test sample as contaminated with mycoplasma in the event that the ratio of (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample) is greater than one.

Claim 34. The method of claim 1, 2 or 33 wherein the method includes a step of

passing the test sample through a filter which retains bacterial cells.

Claim 44. The method of claim 14 wherein the MS reagent is selected from the groups consisting of acetyl phosphate, a precursor of acetyl phosphate, carbamoyl phosphate and a precursor of carbamoyl phosphate.

Claim 45. The method of claim 26 wherein the mammalian cells are adherent cells or adherent primary cells isolated from an animal source.

Claim 46. The method of claim 45 wherein the cells are selected from Vero, MRC5, HUVEC, BSMC, NHEK, MCF-7, AoSMC, A549, HepG2, FM3A, PC12, ARPE-19, CHO and COS cells.

Claim 47. The method of claim 27 wherein the cells are selected from the group consisting of K562, U937, HL-60, Cem-7, Jurkats and leukaemic blast cells

Claim 48. The method of claim 25 where the cell culture is a culture of insect cells.

Claim 49. A method of detecting the presence of contaminating mycoplasma in a test sample comprising:

(i) providing a test sample;

(ii) treating the test sample under a condition sufficient to lyse contaminating mycoplasma but insufficient to lyse bacterial cells;

(iii) detecting and/or measuring the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof in the test sample, and said activity being indicative of the presence of contaminating mycoplasma; and

(iv) identifying the test sample as contaminated with mycoplasma on the basis of detection and/or measurement of said activity in step (iii).

Claim 50. A method of detecting the presence of mycoplasma in a test sample, comprising the following steps:

(i) providing a test sample;

(ii) treating the test sample under a condition sufficient to lyse contaminating mycoplasma but insufficient to lyse bacterial cells

(iii) without adding an exogenous reagent (e.g. substrates for kinase activity) to convert ADP to ATP, detecting or measuring ATP in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement;

(iv) obtaining an ATP and/or light output measurement from a corresponding control sample;

(v) determining the ATP and/or light output measurement ratio as (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample); and

(vi) identifying the test sample as contaminated with mycoplasma in the event that the ratio of (ATP and/or light output measurement from the corresponding control sample)/(ATP and/or light measurement from the test sample) is greater than one.

Claim 51. A method of detecting the presence of contaminating mycoplasma in a test sample comprising:

(i) providing a test sample;

(ii) passing the test sample through a filter which retains bacterial cells;

(iii) detecting and/or measuring the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase, and a mixture thereof in the test sample, and said activity being indicative of the presence of contaminating mycoplasma; and

(iv) identifying the test sample as contaminated with mycoplasma on the basis of the detection and/or measurement of said activity in step (iii).

Claim 52. The method of claim 51, further comprising the following steps performed after step (iii) but before step (iv):

(iiia) obtaining enzyme activity information of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture thereof; detected and/or measured in a corresponding control sample; and

(iiib) comparing the activity detected and/or measured in the test sample in

step (iii) of claim 51 with the activity detected and/or measured in the control sample in step (iiia);

wherein the test sample is identified as contaminated with mycoplasma in step (iv) if the activity detected and/or measured in the test sample in step (iii) of claim 1 with the activity detected and/or measured in the control sample in step (iiia), that is, the ration of the activity detected and/or measured in the test sample in step (iii) to the activity detected and/or measured in the control sample in step (iiia) is greater than one.

Claim 53. The method of claim 51 or 52 wherein detecting and/or measuring the activity of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture thereof in the test sample in step (iii) and/or obtaining enzyme activity information of an enzyme selected from the group consisting of acetate kinase, carbamate kinase and a mixture thereof in a corresponding control sample in step (iiia) comprises detecting and/or measuring the appearance and/or disappearance of one or more of the substrates and/or one or more of the products of the following reactions:

(*Ri*) acetyl phosphate + ADP $\overset{\text{acetate kinase}}{\longleftrightarrow}$ acetate + ATP

(*Rii*) carbamoyl phosphate + ADP $\overset{\text{carbamate kinase}}{\longleftrightarrow}$ ammonia + carbonate + ATP.

Claim 54. A method of detecting the presense of mycoplasma in a test sample comprising the following steps:

(i) providing a test sample;

(ii) passing the test sample through a filter which retains bacterial cells;

(iii) without adding an exogenous reagent (e.g. substrates for kinase activity) to convert ADP to ATP, detecting or measuring ATP in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement;

(iv) obtaining an ATP and/or light output measurement from a corresponding control sample;

(v) comparing the ATP and/or light output measurement ration as (ATP and/or light output measurement from the corresponding control

sample) / (ATP and/or light measurement from the tests sample); and

(vi) identifying the test sample as contaminated with mycoplasma in the vent that the ratio of (ATP and/or light output measurement from the corresponding control sample) / (ATP and/or light measurement from the test sample) is greater than one.

APPENDIX 2. EVIDENCE RELIED UPON

1. Rottem and Barile, "Beware of mycoplasmas," *TIBTECH*, **11**:143-151, (1993) - introduced on page 3, line 26 of specification, complete citation on page 44, item 2). Also introduced by IDS filed August 26, 2004 and considered by Examiner (Bin Shen) on December 14, 2006 (attached to Office Action dated January 18, 2007).

2. McGarrity and Kotani, *The Mycoplasmas,* **Vol IV**, Razin and Barile, Eds., Academic Press, pp. 353-390, (1985) – introduced on page 4, line 27 of specification, complete citation on page 44, item 4). Also introduced by IDS filed August 26, 2004 and considered by Examiner (Bin Shen) on December 14, 2006 (attached to Office Action dated January 18, 2007).

Beware of mycoplasmas

Shlomo Rottem and Michael F. Barile

Mycoplasma infection of cell cultures is widespread and has major detrimental effects on cellular physiology and metabolism. Since cell culture is used extensively, both in research and in industrial production processes, questions of primary concern arise, such as: how can mycoplasma contamination be detected; what are the effects of such contamination on cellular functions; what methods are available for eliminating contamination?

Mycoplasmas are the smallest (0.3–0.8 μm diameter) and simplest prokaryotes. The trivial name mycoplasma encompasses all species included in the class Mollicutes: i.e. the genera *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Anaeroplasma* and *Ureaplasma*. Mycoplasmas lack a rigid cell wall and are incapable of peptidoglycan synthesis; they are thus not susceptible to antibiotics, such as penicillin and its analogues, which are effective against most bacterial contaminants of cell cultures. They are surrounded instead by a single plasma membrane, which has served as an excellent model for studying lipid organization and function in biological membranes[1,2].

Mycoplasmas were first described almost 100 years ago. Yet, despite our long acquaintance with them, their nature and taxonomic status have presented a continuing enigma to microbiologists[3]. Mycoplasmas were originally considered to be viruses because of their small size and their ability to pass through filters with pores of 450 nm, that block the passage of bacteria. Following the discovery of bacterial L-forms, which resemble mycoplasmas in their cellular and colony morphology, it was suggested that mycoplasmas were bacterial L-forms. However, DNA-hybridization studies, and the low G+C content of the mycoplasma genome, ruled out any similarity between mycoplasmas and the majority of bacteria. It is now widely accepted that mycoplasmas evolved from Gram-positive bacteria by degenerative evolution that resulted in a marked diminution in the size of the genome[4,5]. As a result of their small size and the absence of a cell wall, mycoplasmas are pleiomorphic, varying in shape from spherical or pear-shaped cells, to branched-filamentous or helical cells (Fig. 1). Since genome replication is not synchronized with cell division, filamentous forms and chain of beads are frequently observed.

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The limited biosynthetic capabilities of the mycoplasmas make them dependent on their hosts for the supply of many nutrients, hence the difficulty in culturing mycoplasmas in the laboratory. Most species require fatty acids and sterols for growth. The complex media used for culture are usually rich, and contain components such as beef-heart infusion, yeast extract and serum. Defined artificial media have been developed for only a few species[6].

The size of the mycoplasma genome is the smallest recorded for prokaryotes – 600–1700 kb (Ref. 3) – depending on the strain, and with a relatively low G+C content, ranging from 23 to 41%. The small genome size (in some cases, only a quarter that of *E. coli*) should facilitate the development of mycoplasmas as cloning hosts with potential use in biotechnology[7]. However, the genetics of mycoplasmas have remained relatively undeveloped until recently, primarily due to the inadequacy of classical genetic methodology for studying these unusual organisms. Only with the introduction of recombinant DNA (rDNA)-techniques has the direct study of mycoplasma genomes become possible. Although genome analysis, reported to date, has been carried out on only a few species, these appear to be representative of the entire group. Characteristic features include: (1) a small number of genes (e.g. *M. capricolum* requires only 400 genes for all essential functions); (2) all mycoplasma genomes are extremely A+T rich (G+C poor); (3) the organization and structure of essential genes are highly conserved among different species; and (4) deviation from the universal genetic code – the universal termination codon UGA is read by mycoplasmas as a tryptophan codon. This could present problems in expressing mycoplasma genes in other hosts (e.g. *E. coli*), where termination could occur within coding sequences at Trp codons and, conversely, the expression of genes from other organisms in mycoplasmas could result in translational readthrough of termination codons.

There are several recent comprehensive reviews of mycoplasma biology[2,8,9]. This article focuses on an issue of key relevance to biotechnology – the contamination of cell cultures by mycoplasmas.

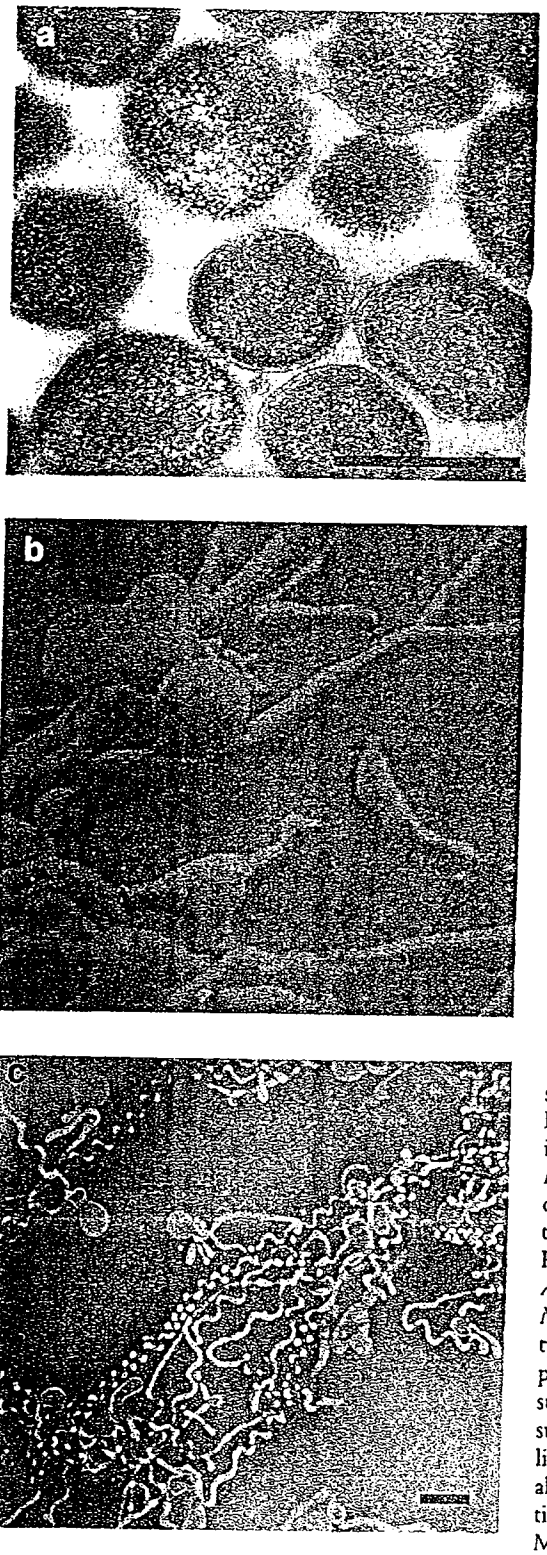


Figure 1

(a) Thin section of helical *M. gallisepticum* cells as seen by transmission electron microscopy. Reproduced, with permission, from Ref. 73. (b) Scanning electron micrograph of filamentous *M. pneumoniae* cells. Reproduced, with permission, from Ref. 74. (c) Helical filaments of *S. citri* preserved by negative staining with ammonium molybdate. Reproduced, with permission, from Ref. 75.

Cell-culture contamination

A mycoplasma was first isolated from a contaminated cell culture by Robinson *et al.*[10]. It is now well established that stable cell lines in continuous culture are frequently contaminated. In studies carried out in the USA at the Food and Drug Administration (FDA), over 20 000 cell cultures were examined during the past 30 years, 15% of which were found to be contaminated – over 3000 mycoplasma contaminants were isolated, detected and speciated[11]. Similar findings have been reported by others[12,13], and even higher incidences of contamination have been reported in other countries. Three different surveys in Japan showed an incidence of mycoplasma contamination of 80% (Ref. 14), an incidence of 65% was reported in Argentina[15] and, more recently, ~32% of the cell cultures examined during the past seven years in Israel were found to be contaminated (S. Rottem and M. Wormser, unpublished).

Contamination of primary cell cultures

In general, primary cell cultures are less frequently contaminated than continuous cell lines. However, since many viral vaccines (such as those for measles, mumps, rubella, polio and rabies) are produced in primary cell cultures, many countries require such cultures to be screened carefully for mycoplasma contamination before approval can be given for release of the vaccine (or other biological intended for human use) to the market-place. Of over 3200 primary-cell-culture lots examined between 1958 and 1972, 42 lots were contaminated, and 51 strains, representing 12 different mycoplasma species, were isolated and identified[11].

Contamination of cell lines

At least 20 distinct *Mycoplasma* or *Acholeplasma* species have been isolated from contaminated cell lines. Ninety-five percent of the contaminants were identified as either *M. orale*, *M. arginini*, *M. hyorhinis*, *M. fermentans* or *A. laidlawii* [11,16], although the frequency of isolation of a particular species varies with the particular study. For example, McGarrity and Kotani[13] isolated many more strains of *M. hyorhinis*, *A. laidlawii* and *M. salivarium*, but far fewer isolates of *M. pirum* or *M. arginini* than found by us[11]. All cell types, including virus-infected, transformed, or neoplastic cell cultures grown in monolayers and/or in suspension, derived from all host-types examined, are subject to contamination. Mammalian and avian cell lines were the most commonly contaminated although, on occasions, cell cultures derived from reptiles, fish, insects or plants were also contaminated. Most studies have examined fibroblast cell cultures, but epithelial, endothelial, lymphocytic and hybridoma cell-culture lines have also been found to be contaminated. The information available on the contamination of cultures of differentiated cell lines is limited, and more data are needed before a proper assessment can be made. However, mycoplasmas have been isolated from, or detected in blood lymphocytes. *M. orale*

was isolated from 'buffy coats' of patients with leukemia[11], and *M. fermentans*, *M. pirum* and uncharacterized species were recovered from lymphocyte cultures from patients with AIDS[17,18].

Sources of contamination

Mycoplasma contamination of vaccines presents a potential health hazard; consequently, identifying the source(s) of contamination is a key concern. The probable source of most mycoplasma contaminants in primary cell culture is the original tissue used to develop the primary cell culture lot. Whereas lung, kidney, or liver tend to be mycoplasma-free, the foreskin, the lower female-urogenital tract, or tumor tissues, are subject to mycoplasma colonization, and generally show a higher rate of contamination[11]. Nonetheless, contamination from exogenous sources also occurs during cell propagation and continuous cell cultures are the most frequently contaminated. A main source of contamination is, in many cases, infection by previously-contaminated cell cultures that have been maintained and processed in the same laboratory[11–14]. Mycoplasmas are spread by using laboratory equipment, media, or reagents that have been contaminated by previous use in processing mycoplasma-infected cell cultures. New cell-culture acquisitions should be quarantined, tested and guaranteed mycoplasma-free before introduction into the tissue-culture laboratory. Common experimental stock materials, such as virus pools, or monoclonal-antibody (mAb) preparations, can also be a key source of mycoplasma contamination. As there is no legal requirement for suppliers to provide mycoplasma-free products, bovine serum should be considered as a possible source of contamination. Mycoplasma contaminants of bovine serum are primarily bovine species, with *A. laidlawii* and *M. arginini* being isolated most frequently[11].

Isolating and detecting contaminating mycoplasmas

Several different approaches are used to isolate mycoplasmas. These include microbiological culture procedures, such as growth on agar and broth culture media; semi-solid agar-broth medium[11], and the large specimen volume (for screening sera or media)[19]; and 'virological type' cell-culture procedures[12,20].

Standard culture procedures

The variation inherent in the undefined, complex media[11,21,22] usually used for *in vitro* culture of mycoplasmas is due to batch variation in compounds such as sera, or yeast extract. Such variation makes the development of defined media attractive. However, a key problem has been the supply of lipids in an available, but non-toxic form, hence, defined artificial media have been developed for only a few species[8].

Most mycoplasmas produce microscopic (100 – 400 μm diameter) colonies with a characteristic 'fried-egg' appearance, growing embedded in the agar, although some (e.g. *M. pulmonis*) may not grow completely embedded, and some freshly-isolated pathogens (e.g. *M. pneumoniae*) produce a more granular, diffuse colony-type. Since they usually grow embedded, mycoplasma colonies can be distinguished from other bacteria by: (1) specific colony shape; (2) being difficult to scrape from the agar surface. Mycoplasmas growing on agar can be identified more specifically by immunofluorescent procedures, using fluorophores conjugated to species-specific antibodies[23].

Cell culture

Some 'non-cultivable' strains cannot readily be grown on standard agar or broth-culture media[20], and cell-assisted culture is required for their isolation. Various non-specific cell-culture procedures have been developed[11,13], and detection of mycoplasma contamination exploits the effects of the mycoplasma on the cultured cells (such procedures resemble the use of cell culture for the detection of viruses). These approaches are particularly useful for the identification and detection of mycoplasma species that adsorb to host-cell surfaces; non-specific stains permit visualization of mycoplasmas adsorbed to cell membranes. In addition, cytadsorbing species have a characteristic infection pattern and cytopathic effects (CPE).

Cell-culture systems are a valuable ancillary tool for the isolation and detection of mycoplasmas and 'indicator-cell culture' procedures using either VERO (African green monkey kidney), or NIH 3T3 cell cultures have been developed (Fig. 2). These cell lines are susceptible to infection by the majority of mycoplasma species and are therefore a reliable 'indicator' system for detecting mycoplasma infection. These procedures[24] are suitable for use with either non-specific systems (for example, non-specific DNA stains; detecting adenosine phosphorylase activity[25]) for detecting mycoplasmas, or in conjunction with mycoplasma-speciation methods (for example, immunofluorescent probes).

Detection methods

Non-specific detection methods that have been reported include staining with DNA-binding fluorochromes, histological stains, electron microscopy and luminol-dependent chemiluminescence[11,13,16]. The non-specific DNA-staining procedure using bisbenzimidazole (33258-Hoechst)[24,26] is simple and inexpensive[12] (Fig. 3). Whereas poorly cytadsorbing mycoplasmas are best detected by growth on agar, or in broth media, DNA staining is effective in detecting cytadsorbing strains. Thus, attempts to detect and isolate an unknown contaminant should use both approaches.

Biochemical identification methods[11,13,16] are based on detecting enzyme activity present in mycoplasmas, but absent, or minimal in uninfected cell cultures. The enzymic activities measured include: arginine deiminase; thymidine-, uridine-, adenosine- or pyrimidine nucleoside phosphorylase; or hypoxanthine- or uracil phosphoribosyltransferase activities[27]. Of these procedures, the adenosine-phosphorylase assay is probably

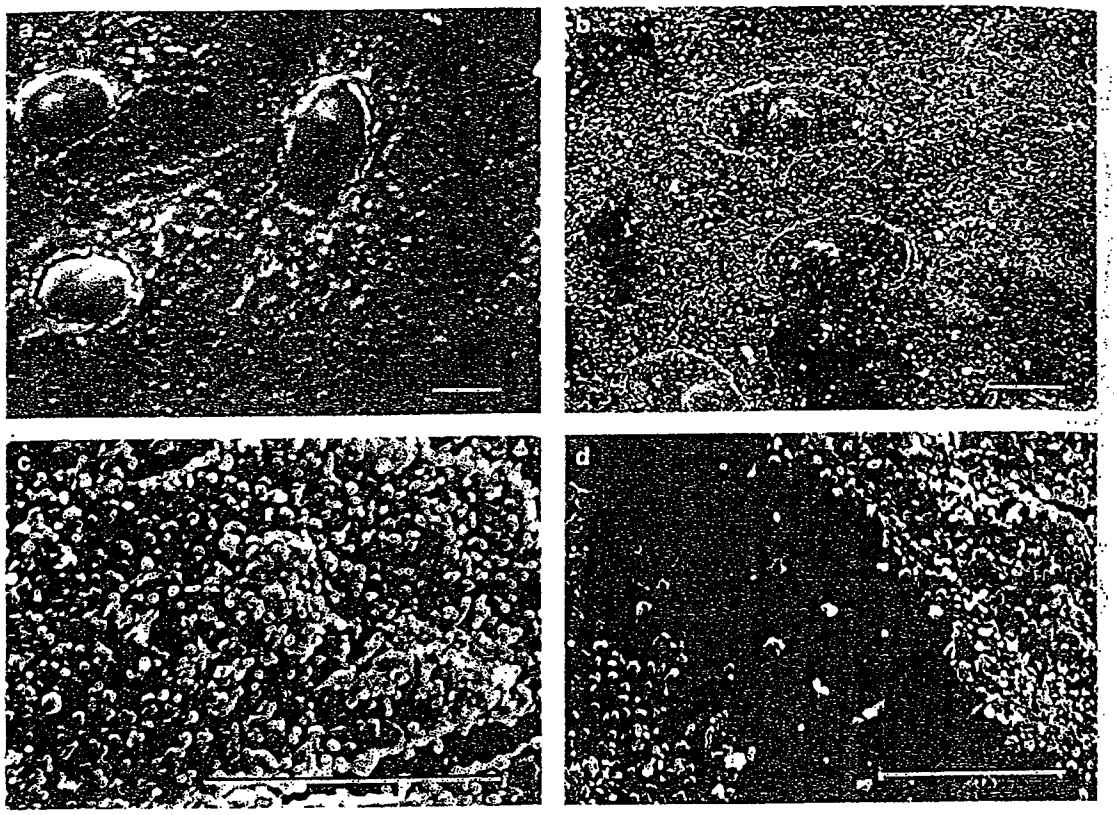


Figure 2
Scanning electron micrographs of VERO (African green monkey kidney) cells. (a) uninfected cells; (b–d) cells infected with *M. fermentans*. (Bar = 10 μm.)

the best, but each method has shortcomings. Procedures based on the isolation and identification of mycoplasmal RNA, or on the comparative utilization of uridine versus uracil in contaminated versus mycoplasma-free cell cultures have also been suggested[28].

Biochemical procedures are most effective for detecting cytadsorbing mycoplasmas, since the infected cells, rather than the culture media, are used for assay. These procedures, however, have several disadvantages: not all mycoplasma contaminants are good cytadsorbing agents, and some possess higher levels of enzymic activity than the host cells. Positive reactions are based on arbitrary values, making low levels of mycoplasma contamination difficult to detect.

The use of DNA probes is increasing steadily. Most of these probes are based on the mycoplasmal ribosomal RNA genes[29], or are synthetic group- and species-specific oligonucleotide probes that are complementary to rRNA[30]. Other genetic probes[31–33] and DNA-hybridization procedures have also been used. However, such systems are still at an early stage of development and are currently less informative than culture techniques. Barile *et al.*[34] first reported the use of immunofluorescence to detect and identify mycoplasmas in contaminated cell cultures. A number of other immunofluorescence procedures have also been reported using species-specific polyclonal antisera[35], or monoclonal antibodies, conjugated with fluorescein or peroxidase. Gabridge *et al.*[36] detected and speciated common cell-culture mycoplasmas using an enzyme-linked-immunoadsorbent assay with biotin–avidin amplification on solid-phase microporous membranes. Other investigators have used immunobinding onto nitrocellulose paper[37], or combinations of specific and non-specific staining procedures[38].

Regulatory requirements for human biologics

Currently, the recommended test requirements for biologics in the USA and in some other Western countries are as follows: (1) The master- and working-cell seed banks must be free of mycoplasmas. (2) The product-harvest concentrates must be free of mycoplasmas. (3) All products produced in cell substrates, a generic term used for all tissue cells grown *in vitro*, must be tested. This includes viral vaccines (such as poliovirus, adenovirus, measles, rubella, mumps and rabies), monoclonal antibodies, immunological modifiers and cell-culture-derived blood products, such as tissue-type plasminogen and erythropoietin (EPO). In brief, the harvest concentrate is inoculated onto agar medium and into broth that is subcultured periodically onto agar media. The indicator-cell-culture system is also included in each test. An equivalent procedure is acceptable if detailed data presented to the FDA demonstrates that it is equal to, or better than, the recommended procedures. The current test require-

ments and the 'Points to Consider' for biologics marketed in the USA can be obtained from the Division of Bacterial Products, OBR, FDA, Bethesda, MD 20892, USA.

Effects of mycoplasma infection on cell cultures

Effects on cell function and metabolism

Mycoplasmas have long been recognized as common contaminants, capable of altering the characteristics of cultured cells. The nature of the effects depends on the contaminating species and strain of mycoplasma, and on the type of cell infected. Many *Mycoplasma* species produce severe cytopathic effects (CPE), whereas others produce very little overt cytopathology, and covert contamination may go undetected for months. The biological and biochemical activities of the mycoplasma determine the effect on cells and the degree of CPE.

Fermenting mycoplasmas degrade simple sugars rapidly and generate copious amounts of acidic metabolites that alter cell functions and/or produce severe CPE. All mycoplasmas require nucleic acid precursors (free bases, nucleosides, or nucleotides), amino acids and fatty acids. In addition most mycoplasmas have an absolute requirement for sterols[1]. Mycoplasmas use either arginine or dextrose (seldom both) as an energy source. The growth of mycoplasmas that use arginine as an energy source[39] may deplete the medium of arginine rapidly, thus depriving the cell culture of an essential amino acid. Arginine depletion can affect protein synthesis, and cell division and growth. It can also inhibit or stimulate lymphoblast proliferation and viral replication. Attachment of a mycoplasma to a cell can alter or disrupt the integrity of the host-cell membrane, causing the cells to be leaky. Frequently, the number of mycoplasmas far exceeds (often by 1000-fold) the number of tissue-culture cells in an infected cell culture. Mycoplasmas compete effectively with tissue-culture cells for medium nutrients, thus depriving the cells of essential nutrients, resulting in profound effects on cell metabolism and function[1,45].

Perez *et al.*[40] observed that the incorporation of nucleic-acid precursors in mycoplasma-infected mammalian cell cultures is altered. Hellung-Larsen and Fredriksen[41] reported similar effects on the incorporation of different precursors into the RNA components of infected cell cultures, due to precursors being incorporated into mycoplasmal RNA rather than host-cell RNA. *M. pulmonis* affects protein and glucosaminoglycan synthesis in infected connective-tissue cells[42]. *M. orale* induces secretion of murine types I and III collagenase in infected NIH 3T3 cell cultures[43]. Crowell *et al.*[44] suggested that mycoplasma attachment to infected cell membranes interferes with membrane-receptor function, or alters signal transduction, thus inhibiting the cellular autocrine response. Hatcher[45] reported that mycoplasma-infected cells secrete larger amounts of tissue plasminogen activator (tPA), and suggested that this activity may play a role in tissue destruction in mycoplasma disease states.

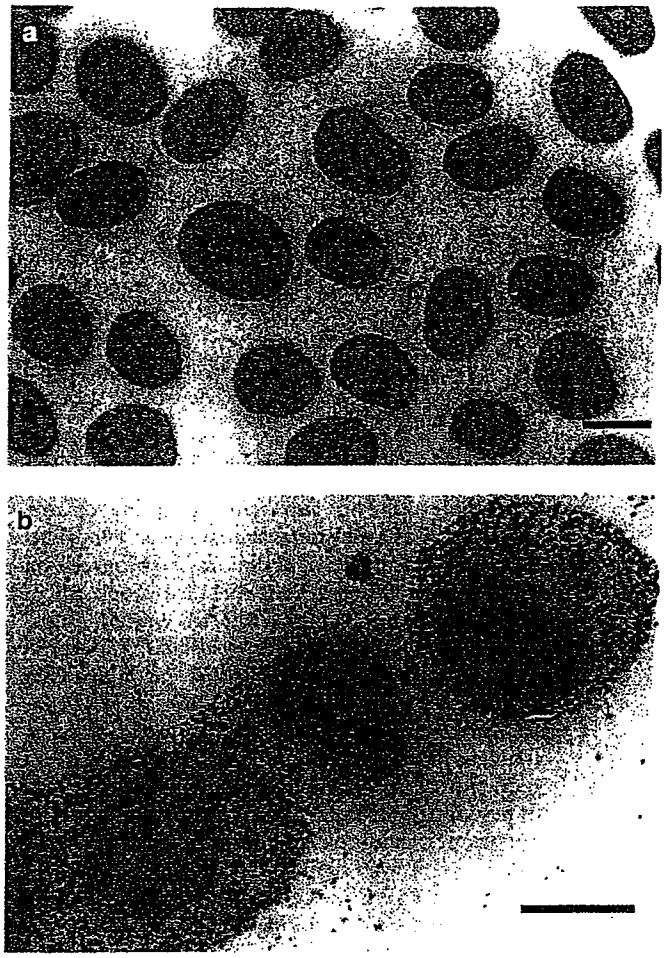


Figure 3

VERO cell culture infected with *M. hyorhinis*, stained with the DNA stain Hoechst 33258. (Bar = 10 μm.)

Effects on morphology

- *Covert infection*. Contamination may go undetected because mycoplasma infections do not produce the overt turbid growth that is commonly associated with bacterial and fungal contamination. The morphological cellular changes may be minimal or unapparent. Frequently, the cellular changes are similar to those caused by nutrient deprivation, such as depletion of amino acids, sugars, or nucleic-acid precursors. These morphological effects can be reversed by changing the medium, or by replenishing the medium with fresh nutrients.

- *Overt effects*. Collier[46] was one of the first to report mycoplasma-mediated CPE. Affected cultures are characterized by stunted, abnormal growth and rounded, degenerated cells and a macroscopically 'moth-eaten' appearance at the edge of the monolayer. Certain strains of *M. arginini* lyse cells in some, but not all, human lymphoblastoid cell cultures, and the addition of arginine to the medium prevents lysis. The large amounts of acid metabolites produced by fermenting mycoplasmas reduce the pH of the medium

and cause the cell monolayer to detach from the culture-vessel surface.

Mycoplasmas attached to cells release toxic, enzymic and cytolytic metabolites directly onto the cell membrane. Some mycoplasmas selectively colonize defined areas of the cell monolayer, resulting in the formation of microcolonies, microlesions and small foci of necrosis[47]. Microcolonization suggests that mycoplasma-specific receptors are localized in defined areas of the cell monolayer. However, other mycoplasmas, such as *M. hyorhinis*, attach to every cell, producing a generalized CPE and destroying the entire monolayer.

Chromosomal aberration

Arginine-utilizing and fermenting mycoplasmas may induce chromosomal aberrations *in vitro*. These have been observed in: (1) human amnion-cell cultures infected with an unspeciated mycoplasma; (2) human diploid W1-38 cells, infected with *M. orale*, *A. laidlawii*, *M. hyorhinis* or *M. pulmonis*; (3) hamster fibroblasts infected with *M. salivarium*; and (4) human-lymphocyte cultures infected with *M. salivarium*, *M. fermentans*, *M. arthritidis*, or ureaplasmas[11,13]. Chromosomal breakage, multiple translocation events, reduction in chromosome number and the appearance of new and/or additional chromosome variants are the commonest induced changes. Since histones are arginine-rich, it was suggested that mycoplasmas may exert their effects on cellular genomes by depleting arginine and thus inhibiting histone synthesis. However, as fermenting mycoplasmas and ureaplasmas also induce chromosomal aberrations, other mechanisms, including competition for nucleic acid precursors, or degradation of host-cell DNA by mycoplasma nucleases, must be involved. Mycoplasma nucleases have been isolated from contaminated cell cultures[48].

Although mycoplasmas can induce chromosomal aberrations *in vitro*, attempts to induce tumor formation in animals have been uniformly unsuccessful. Mycoplasmas can inhibit viral transformation of cell cultures by known oncogenic viruses; *M. orale* inhibits the effects of Rous sarcoma and Rous-associated viruses in chick embryo fibroblasts. Other mycoplasma contaminants reduce the number of foci in simian SV40- and polyoma-infected cell cultures[11,13,16].

Virus propagation in cell cultures

Some mycoplasmas have no detectable effect on viral growth. Others can decrease, or even increase, virus yields in infected cell culture[11,13]. The effect depends on the strain or species of mycoplasma, the virus, and the cell culture used. At least two mechanisms responsible for decreasing viral yields *in vitro* have been identified. The cytolytic, fermenting mycoplasmas suppress metabolism and growth, resulting in severe CPE and a decrease in viral yields. Arginine-utilizing mycoplasmas decrease the titers of arginine-requiring DNA viruses (including herpes simplex virus[49], vaccinia virus[50], SV40 virus, adenovirus types 1, 2 and 5, polyoma virus, and human and simian cytomegaloviruses[1,13]) by depleting arginine from the medium. Changing the medium or replenishing arginine reversed the effect. Measles-virus titers were decreased by either *M. hyorhinis*, a cytolytic fermenter, or by various non-fermenting, arginine-utilizing mycoplasmas. Thus, reduction in titer can be caused by more than one mechanism. The immunoreactivity of varicella zoster virus was also reduced in mycoplasma-infected cell cultures[51]. Scott *et al.* showed that previously reported immunosuppressive effects by cytomegalovirus were due to mycoplasma contamination and not the virus.

Mycoplasmas can increase virus yields by inhibiting interferon induction and interferon activity. Singer *et al.*[53] showed that *M. arginini* or *M. hyorhinis* inhibit interferon production, interferon activity, and cellular resistance to viral infection, resulting in increased yields of Semliki Forest virus (SFV). Mycoplasmas may also render cell cultures less sensitive to exogenously supplied interferon[11] and can, as a consequence, affect the apparent virus titers obtained by the standard cell-culture interferon assay[54]. A particular *Mycoplasma* species can affect cell cultures in several different ways. *M. hyorhinis* can produce CPE and reduce virus yields; however, if the CPE is suppressed by changing the medium, it can inhibit interferon production and increase virus yields. This phenomenon can be used to advantage by exploiting the decreased interferon induction and activity due to mycoplasma infection to increase titers of latent, interferon-sensitive viruses[11].

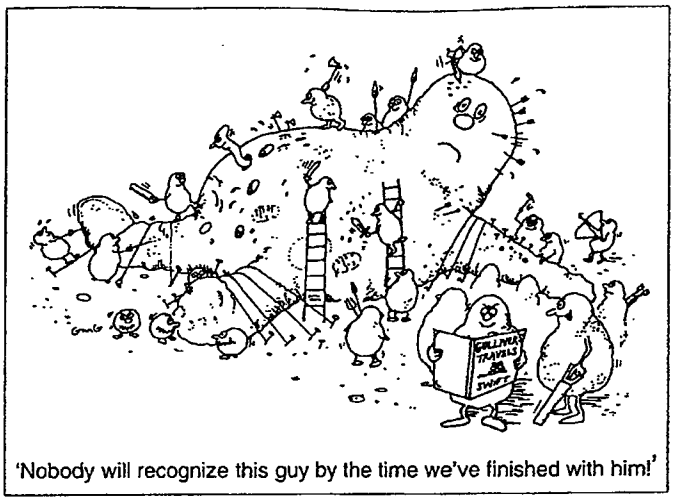
Induction of interferon activity

Interferon expression can be induced by mycoplasma infection in both cell cultures and animals. Beck *et al.*[55] induced interferon by infecting mouse spleen-cell cultures with mycoplasmas. Mice inoculated with a strain of *Acholeplasma* were protected against infection with SFV, and resistance to infection was mediated by the induction of interferon. Lipoglycans present in some *Acholeplasma* species have endotoxin-like properties that induce interferon expression in mice. Some species induced an early response in mice (six hours after inoculation), while other species produced a delayed response. Conversely, viable or non-viable sonicated preparations of various *Mycoplasma* and *Acholeplasma* species suppressed the interferon response to Newcastle disease virus in mice. Prior exposure to mycoplasmas can either suppress, or enhance a virus infection in mice[56].

Effect on viral infections

Mycoplasmas can also alter the progress of viral infections in organ cultures or animals (see Refs 11 and 12 for citations) with dual infections (mycoplasma and virus) causing more damage than infection by either individual agent. Because mycoplasmas cause destructive virus-like CPE, many investigators have mistaken cytolytic mycoplasmas for viruses[11]. Like viruses, mycoplasmas are filterable, hemadsorbant, hemagglutinant, resistant to certain antibiotics, inhibited by

antisera, able to induce chromosomal aberrations, and sensitive to detergents, ether and chloroform. Kapikian *et al.*[57] showed that the reputed 'Crohn's disease agent' was a *M. hyorhinis* contaminant, and the first established mycoplasma pathogens of humans (*M. pneumoniae*) or animals (*M. mycoides* subspecies *mycoides*) and plants were all first believed to be viruses. Experimental identification may also prove difficult: Sydiskis *et al.*[58] reported that a mycoplasma contaminant co-sedimented with mouse mammary tumor virus (MMTV) in sucrose density gradients. Thus, the virologist must be aware of mycoplasmas and their properties to avoid misinterpreting data.



'Nobody will recognize this guy by the time we've finished with him!'

Effects on lymphocytes

Biberfeld and Gronowicz[59] reported that *M. pneumoniae* can activate mouse B cells, and because the mitogenic component was heat-stable, it was postulated to be endotoxin-like. Some *Mycoplasma* and *Acholeplasma* species possess membrane-bound lipoglycans which also have endotoxin-like activity[60]. The lymphokine-like activity of a *Mycoplasma arginini* strain was reported to enhance immunoglobulin secretion[61] and Proust *et al.*[62] showed that a soluble 'lymphokine-like product' in the serum-free supernatant of a T-cell hybridoma induces proliferation and maturation of B cells as a consequence of mycoplasmal contamination. Other mycoplasmas were shown to alter the Fc receptors for IgE of rat basophilic leukemia cells[63].

Effect on macrophages

Mycoplasmas can affect a variety of macrophage activities. The differential induction of bone-marrow macrophage proliferation by some, but not all, mycoplasmas involves granulocyte–macrophage-stimulating factor (GM-CSF)[64]. Several reports have shown that mycoplasmas induce macrophage-mediated cytolysis of neoplastic cells[65,66], by tumor necrosis factor (TNF). Several *Mycoplasma* and *Spiroplasma* species are very efficient TNF-inducing agents, activating bone-marrow macrophages to secrete very high levels of TNF-α and to mediate tumor cytolysis[67,68]. The capacity to induce macrophage TNF-α secretion resides exclusively in the cell membrane, apparently associated with a low-molecular-weight membrane protein. Mycoplasma membranes and lipopolysaccharide act synergistically to augment TNF-α secretion by C57BL/6-derived macrophages, and lipopolysaccharide-unresponsive C3H/HeJ-derived macrophages were also activated by mycoplasma membranes which do not contain lipopolysaccharide. These findings suggest that the mechanism by which mycoplasma membranes activate macrophages differs from that of lipopolysaccharide. Further studies showed that human monocytes also secrete TNF-α following activation by mycoplasma membranes. Hommel-Berrey and Brahmi[69] detected soluble cytotoxic factors generated by mycoplasma-contaminated target cells. They discussed the significance and relevance of infection on natural killer (NK) cell-mediated killing. Arai and colleagues[70] showed that the supernatants of mycoplasma-infected macrophage cultures contained a potent cytotoxic activity to TNF-α-sensitive L-cells, but not to insensitive L-cells. They suggested that the mycoplasma-induced TNF-α activity might be responsible for the enhanced cytotoxic activity of macrophages and could also induce resistance to mycoplasma infections in the host[70].

Elimination and prevention of mycoplasma contamination

Ever since mycoplasma contamination of cell cultures was first reported, attempts have been made to develop methods for the elimination of mycoplasmas, including the use of antibiotics such as tetracycline, kanamycin, novobiocin, tylosin, gentamycin, doxycycline, thiayline and quinolones; surface-active agents; and the use of anti-mycoplasma antisera[11,13]. Many of the methods were unreliable. Some techniques may apply to some, but not all, mycoplasma species; some of them are laborious and/or time consuming. It was suggested, therefore, that whenever possible, the infected cell culture should be discarded and replaced with a mycoplasma-free culture[13]. When the cell culture is irreplaceable, the use of antibiotic mixtures, detergents, prolonged heating treatments (40–42°C), treatment with specific antisera, or the combined use of high-titer, specific, neutralizing antisera and a high concentration of a pre-tested antibiotic[11,13] are the commonest approaches. One has to keep in mind that cell-culture contaminants that have been continuously exposed to antibiotics develop resistance to the drug, and antibiotic-resistant strains have been isolated for most mycoplasma species tested. Treatment may also induce the selection of a subpopulation of cells and the treated cell culture may differ in its characteristics from the original culture.

Elimination of mycoplasmas from contaminated cell cultures by passage through nude mice[71] has been successful for some, but not all, mycoplasmas, and by some, but not all, investigators. Animals have a rich oral and/or urogenital mycoplasma flora. Consequently, mycoplasmas are frequently isolated from

infected or neoplastic tissues. They have also been recovered from exudates or ascites, and especially from immunosuppressed humans or animals. Passage through animals could conceivably contaminate the test-cell culture with the indigenous mycoplasma flora. Trauma and other stressful conditions permit mycoplasmas and other agents to gain entry and infect the peritoneal cavity.

Twelve years ago, we described the selective killing of mycoplasmas from contaminated cell culture[72]. The method is based on the selective incorporation of 5-bromouracil (5-BrUra) into mycoplasmas, and the induction of breaks by light in the 5-BrUra-containing DNA. This photosensitivity was greatly increased by the binding of the fluorochrome 33258-Hoechst to the DNA. The unusually high content of A+T makes the mycoplasma DNA an excellent candidate for the induction of breakage by the combined action of 5-BrUra, 33258-Hoechst and visible light.

The measures used successfully for prevention of contamination are designed to control the sources and the spread of contamination. They are based on good laboratory practices[11,13] and are summarized in detail in Ref. 16.

Conclusions

Mycoplasma infection is one factor that substantially affects the biological properties of cells *in vitro*. As the use of cell cultures is widespread, not only in research laboratories, but also in the expanding biotechnological industry, one of the primary concerns of cell biologists is whether or not a cell culture is infected by mycoplasmas; what the effects of such infection on the cell culture are, and what the methods of eliminating the infection are. In this review, we have presented a broad overview of mycoplasma–cell interactions, discussing mycoplasma infection and contamination of cell cultures; the effects of infection on cell function and activities, and the common procedures for isolation, identification and speciation of mycoplasmas. It is especially important to emphasize that mycoplasma contamination can affect virtually every parameter and every function and activity of a cultured cell. The prudent investigator must be aware of this and should maintain constant vigilance for the presence of contamination in order to properly interpret data.

Acknowledgements

We thank P. Freedman for technical assistance and M. Tarshis for his suggestions and comments.

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book reviews

Protein crystals – more matter and less art

ystallization of Nucleic Acids and Proteins – A Practical Approach

edited by A. Ducruix and R. Giegé, IRL Press at OUP, 1992. UK£25.00 (xxiv + 331 pages) ISBN 0 19 963246 4

Advances in biotechnology and macromolecular engineering over the past decade have resulted in a large increase in the number of biological macromolecules available for structural studies. Modern instrumentation for X-ray analysis, combined with high-speed computers, have revolutionized data collection and structure solution in X-ray crystallography. The rate-limiting step in any crystallography project is the production of crystals that diffract to high resolution.

For many years the crystallization of biological macromolecules has been regarded as 'an art, rather than a science', due to unpredictable, and often irreproducible results. Successful crystal growers are often regarded as having 'green fingers'. From my own experience of protein crystallization, I would say that dogged perseverance is a necessary requirement for successful crystallization, coupled with a measure of intuition and good luck. However, in recent years the crystallization of macromolecules has been put on a more rational basis with the emergence of the new discipline of biocrystallogenesis. This discipline covers the biology, biochemistry, physics and engineering aspects of macromolecule-crystal growth. This book in the *Practical Approach* series is an invaluable contribution to the literature in this research area.

The major aim of the book is to present the methods used to obtain crystals of biological macromolecules, and although it is intended to be read by a wide range of scientists, it will be most useful for students and beginners in the field of crystallization. Detailed laboratory protocols are given throughout the book with reference to the theoretical concepts and principles underlying them. The first chapter provides an introduction to the crystallogenesis of biological macromolecules, describing the general principles and giving a brief historical survey. This is followed by a chapter on the preparation and purification of macromolecules for crystallization – a topic of paramount importance when initiating a crystallization project. The success, or failure, in

10 / CELL CULTURE MYCOPLASMAS

Gerard J. McGarrity and Hitoshi Kotani

I. Introduction 353
Characteristics of Mycoplasma-Infected Cell Cultures 355

II. Effects of Mycoplasmas on Cell Cultures 357
A. Effects on Growth and Morphology 358
B. Effects on Viruses 360
C. Tumor Viruses 362
D. Cytogenetic Effects 363
E. Effects on Mutagenic Assays 364
F. Effects on Nucleic Acid Metabolism 366
G. Effects on Carbohydrate Metabolism 367
H. Effects on Lymphoblastoid Cells in Culture 368
I. Effects on Interferon 370
J. Isoenzyme Studies 372
K. Specialized Cell Culture Systems 373
L. Spiroplasma and Ureaplasma Infection of Cell Cultures 374

III. Methods of Detection 375
A. Microbiological Medium 376
B. Indirect Detection Methods 377
C. DNA Staining 378
D. Mycoplasma-Mediated Cytotoxicity 379
E. Biochemical Detection Methods 380
F. Electron Microscopy 382

IV. Methods of Elimination 383

V. Methods of Prevention 384
References 386

I. INTRODUCTION

Cell biologists are primarily interested in three practical questions regarding mycoplasmas: (1) Are they present? (2) If they are present, what effect do they

THE MYCOPLASMAS, VOL. IV

have on specific experimental systems? and (3) If present, how does one either get rid of them or obtain a cell culture free of them? Mycoplasmologists, of course, are purists. They want proper diagnostic and identification procedures. Both perspectives are valid. With the increased use of *in vitro* techniques to study cell physiology and molecular biology, and with the rapid advances in mycoplasmology, an in-depth review of the interactions of mycoplasmas with vertebrate and invertebrate cells *in vitro* can offer a valuable insight for these fields as well as for prokaryotic–eukaryotic cell interactions.

Some cell biologists are convinced that mycoplasmas have been standing idly by throughout evolution awaiting the development of cell cultures to find their true evolutionary niche. The first report of mycoplasmas present in uninoculated cell cultures came in 1956 by Lucille Robinson and colleagues. These workers were studying the effects of mycoplasmas on HeLa cells; mycoplasmas were isolated from their negative controls. The organism was not identified, and it is unknown whether the isolate was the same as the organism added to the experimental system. Procedures were not available in 1956 to speciate the isolate as most of the organisms now known to infect cell cultures were not speciated at that time. In fact, our present knowledge about media and other conditions for isolation of mycoplasmas from cell cultures suggests that the isolate was a nonfastidious mycoplasma that could be isolated under aerobic conditions, e.g., *Acholeplasma laidlawii*.

Other workers in the late 1950s and early 1960s showed that the presence of mycoplasmas in cell cultures was far more extensive than expected from the single report of Robinson *et al.* (1956). Using microbiological and serological procedures, workers demonstrated that a significant number of cell cultures contained mycoplasmas (Hayflick, 1965). Hayflick (1969) stated that much of the results of cell culture investigations of the 1950s and 1960s should be viewed skeptically because of mycoplasma infection.

While it is tempting to quote figures regarding the incidence of mycoplasmal infection (MI) of cell cultures, such figures can be misleading. Incidence of infection is significantly influenced by the population of cultures being surveyed and by the quality control procedures practiced by the individual laboratory. Our laboratory has performed in-depth surveys for many laboratories that utilize large numbers of cell cultures. Some laboratories have virtually no mycoplasmal infections; their quality control procedures either prevent it, or, if it does occur, detect it at an early stage before it spreads to other cell cultures in the laboratory. In other laboratories where appropriate controls are lacking, the incidence can approach 100%; only those cultures recently introduced into the laboratory remain uninfected, at least for the time being. Therefore, figures from small, limited surveys can be biased, depending on the nature of the laboratories included. A further unknown is that many, perhaps most, cell culture laboratories may not assay for cell culture mycoplasmas. This failure would tend to reduce

TABLE I. **Reported Incidence of Mycoplasmal Infection of Cell Cultures**

Reference	Number of infected cultures/tested cultures (% infected)
Del Giudice and Hopps (1978)	2,452/17,666 (13.8)
Barile *et al.* (1978)	2,800/17,000 (16.5)
McGarrity (unpublished)	1,027/20,309 (5.1)
Polak-Vogelzang (1983)	478/1,469 (27.0)
Koshimizu and Kotani (1981)	78/90 (87.0)

the overall incidence. Table I lists the results of a number of published surveys. Our laboratory assays younger cell cultures, passage 5 or less, and cultures submitted for deposition in cell repositories. Cultures found to contain mycoplasmas are destroyed. Mycoplasma-free cell cultures are tested two to four times during the characterization process. This selectivity decreases overall incidence. The citation of Koshimizu and Kotani (1981) is a summary of three different surveys in Japan. An incidence of 80% was reported. The current incidence for continuous cell lines in the United States probably is closer to the figures of approximately 15% cited by Barile *et al.* (1978), Barile (1979), and Del Giudice and Hopps (1978).

Primary cell cultures do not have a high incidence of MI; it is of the order of 1%. Care should be taken, however, in establishment of cell cultures from body sites that can be colonized with mycoplasmas *in vivo*, such as respiratory and genital tracts and blood of immunosuppressed patients. Cell cultures derived from tissues colonized with mycoplasmas *in vivo* should be assayed during early passage. With these exceptions, the tissues used to establish cell cultures are not a major source of cell culture mycoplasmas. This can also be documented by the virtual lack of murine and avian mycoplasmas in the large number of mouse, hamster, and chick cell cultures being used all over the world.

Characteristics of Mycoplasma-Infected Cell Cultures

Although many reports still describe the presence of mycoplasmas in cell cultures as "contamination," the term is inaccurate and misleading. The presence of mycoplasmas and their parasitism on the host cell culture represents a true *in vitro* infection. As with other infectious diseases, a focus of infection must be treated and cured, or destroyed. Otherwise it can serve as a source of further infection, endangering the healthy members of the population. Cell biologists must also recognize the true biochemical nature of infected cell cultures. Table II presents some general features of mycoplasma-infected cell cultures. The number of mycoplasmas per milliliter of cell culture supernatant is based on dozens of measurements in our laboratory. Other numbers represent estimations

TABLE II. Generalized Properties of a Mycoplasma-Infected Cell Culture

Property	Value
Number of mycoplasmas/ml supernatant medium	10^7-10^8
Maximum number of mycoplasma gene products	550[a]–1100[b]
Mycoplasma DNA/host cell DNA	15–30%
Mycoplasma protein/host cell protein	25%
Effect on cell culture	Unpredictable

[a]For *Mycoplasma* species.
[b]For *Acholeplasma* species.

based on calculations in our (McGarrity *et al.*, 1980a) and other publications (Hamet *et al.*, 1980; Razin and Razin, 1980).

In a detailed review, Barile *et al.* (1978) list 17 different mycoplasmas isolated from cell cultures. However, four mycoplasmas are responsible for 90–95% of all reported infections: *A. laidlawii*, *Mycoplasma orale*, *M. hyorhinis*, and *M. arginini*. The percentages of these are listed in Table III. *Mycoplasma salivarium* represented a significant number of isolates in our survey; however, all but one of these was from a single laboratory. Otherwise, our data for species isolated from cell cultures parallel those of Barile *et al.* (1978), Barile (1979), and Del Giudice and Hopps (1978).

The origins of mycoplasmal infection of cell cultures are bovine serum (*A. laidlawii* and *M. arginini*) and laboratory personnel (*M. orale*). *Mycoplasma hyorhinis* is a porcine species, but probably is not introduced through trypsin, which is derived from porcine stomachs. In fact, inoculation of *M. hyorhinis* into trypsin inactivated the organism (McGarrity *et al.*, 1979a). *Mycoplasma*

TABLE III. Percentage of Cell Cultures Infected by *Acholeplasma* and *Mycoplasma* Species

Species	Percentages infected	
	Del Giudice and Hopps (1978)	McGarrity *et al.*[a]
A. laidlawii	8.4	15.0
M. hyorhinis	22.9	40.5
M. orale	29.6	28.6
M. arginini	23.8	5.0
M. salivarium	0.1	7.0
M. pirum	7.5	0
Others	7.7	3.9

[a]Current results from this laboratory.

hyorhinis may be introduced via bovine serum, as is *A. laidlawii* and *M. arginini* (Barile and Kern, 1971). Cattle and pigs are sometimes processed in the same abattoirs, and cross-contamination is a possibility. Barile and Kern (1971) isolated *M. hyorhinis* from bovine serum.

While bovine serum and laboratory personnel are the original sources of cell culture mycoplasmas, infected cultures themselves are the biggest source of cell culture infections. This is due to the large concentrations of mycoplasmas in infected cell cultures, the relative ease of droplet formation during handling of cell cultures, and the relative resistance of mycoplasmas to dehydration. Kundsin (1968) and Furness *et al.* (1967) showed that mycoplasmas could survive aerosolization, depending on the mycoplasma species and relative humidity. We recovered *A. laidlawii* from work surfaces 7–10 days after inoculation (McGarrity, 1976).

II. EFFECTS OF MYCOPLASMAS ON CELL CULTURES

A vast amount of literature exists on the effects that mycoplasmas have on their cell culture hosts. Many of these published studies were unplanned in the sense that MI was discovered after the study was completed. An additional set of controls could be performed to attempt to salvage part of the study. In this way, the effect of mycoplasmas on a given parameter of cell biology could be published. This has resulted in more nonspecialists publishing in the field than in probably any other area of cell biology. While this has made important contributions, there have been problems. Often, MI has not been adequately confirmed, the mycoplasma isolate was not identified, or other controls were lacking. This is especially true in reports suggesting a specific effect of MI be used as a basis of detecting cell culture mycoplasmas.

Given these limitations, one can still present a review of the effects and potential effects of MI of cell cultures. Publications of various effects of MI tended to follow the opening up of cell cultures to new specialty areas. In the 1960s, many papers focused on the effects on viral and interferon titers. In the 1970s, as geneticists and cytogeneticists began to employ *in vitro* techniques, publications on effects of MI in these areas began to appear. More recently, there have been reports on mycoplasmal effects on isoenzyme studies and lymphoblastoid cells.

Other reviews of effects of MI have been published. Most notable are those of Stanbridge (1971), Barile *et al.*, and Barile (1979). These have been updated (Stanbridge and Doerson, 1978; Barile and Grabowski, 1978). A review of the cytogenetic effects of MI on cell cultures has been published (McGarrity *et al.*, 1984a). The purpose of the present review is to offer a comprehensive picture of the effects of MI, especially in those areas not covered by past reviews.

A. Effects on Growth and Morphology

The observed effects of MI in cell cultures can be due to mycoplasma gene products, enzymes, toxins, etc., to mycoplasmal utilization of media or host cell components, or to the secondary effects of mycoplasma growth, e.g., pH. As seen in Table II, *Mycoplasma* species were estimated to produce about 550 gene products; *Acholeplasma* species, having a genome about twice the molecular weight of that of mycoplasmas, can produce a maximum of 1100 gene products. However, as Razin and Razin (1980) point out, the low guanine + cytosine ratio of mycoplasmal DNA probably places further restrictions on the number of gene products. Mycoplasmas do not typically produce toxins *in vivo* to enhance their pathogenicity. Some mycoplasmas produce hydrogen peroxide, and this can have a direct effect on cells. For example, Lanks and Chen (1979) showed that H_2O_2 produced by mycoplasmas yielded false positive results in immunoperoxidase assays.

The four mycoplasma species routinely isolated from cell cultures do not exhibit attachment organelles. Different species, or even strains of the same species, adsorb to cells in varying degrees. In transmission electron micrographs, Phillips (1978) has observed a space of some 50 Å containing fibrous material separating the cultured cells from the mycoplasmas adsorbed to their surface. Often the plasma membrane of the host cell is involuted slightly to fit the contours of the infecting mycoplasma and is more electron dense in this area.

A more significant general mechanism to explain the effects of MI is mycoplasmal utilization of medium components or supplements, which renders them unavailable to the host cells. Because of the variety of mycoplasma species, strain differences, different cell cultures, cell culture media and supplements, one cannot predict even the generalized effects of MI. *Acholeplasma laidlawii* and *M. hyorhinis* are fermentative species. *Mycoplasma orale* and *M. arginini* are not. *Mycoplasma orale, M. arginini,* and *M. hyorhinis* require sterols for growth; *A. laidlawii* does not. *Mycoplasma hyorhinis* tends to adsorb to cultured cells more than the other three species. Other mechanisms are also possible. Zucker-Franklin *et al.* (1966) showed mycoplasmas attached to HeLa cells and suggested that mycoplasma induced large increases in surface area, including formation of long cytoplasmic processes and membrane damage.

Considering the large concentrations of mycoplasmas in infected cultures, one would think that infection would invariably lead to cytotoxicity. Not always. When cytotoxicity is produced, it can be due to effects such as acid pH due to sugar fermentation by *A. laidlawii,* depletion of an essential medium nutrient such as arginine by arginine utilizers, or by action of mycoplasma-produced hydrogen peroxide, among others.

Cytotoxicity can be the first suggestion of MI. It can be a transient toxicity which is eliminated by refeeding or passaging the culture. This is typically

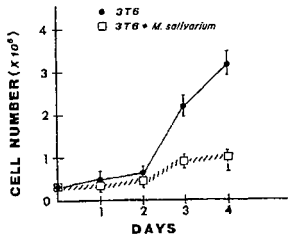

FIGURE 1. Effect of *M. salivarium* on growth rate of 3T6 mouse embryo fibroblasts. From McGarrity *et al.* (1980a).

observed with toxicity induced by arginine utilizers. The organisms deplete the medium of essentially all the arginine within 2–3 days (Barile and Grabowski, 1978). After refeeding, when the arginine concentration returns to normal, no evidence of toxicity is apparent. Stanbridge (1971) has shown that of all the amino acids in cell culture media, only arginine is significantly depleted by MI. Ornithine, the product of the arginine deiminase pathway, exhibits increased concentrations in cell cultures infected with arginine-utilizing mycoplasmas.

In many instances, however, no toxicity accompanies MI. This makes MI less apparent and stresses the urgency for regular assays to detect MI. In a few instances we have observed that infected cultures actually appear healthier than their noninfected counterparts. These instances have occurred in human cell cultures derived from patients with genetic defects. Perhaps the mycoplasmas are supplying an enzyme or substrate that is absent or deficient in these cultures.

Our studies on *M. salivarium* strain VV (McGarrity *et al.*, 1980a) showed it to have no adverse effects on growth of a human lymphocyte culture, GM-130, during the first five passages after infection. The same strain produced a 60% decrease in growth in 3T6 mouse fibroblasts (Fig. 1). These differences were observed despite the fact that the concentrations of *M. salivarium* were greater than 10^8 CFU/ml supernatant in both cultures. In unpublished studies, we have routinely carried 3T6 cultures infected with various mycoplasmas. One strain, *A. laidlawii* MG, produced extensive cytotoxicity, reduced growth, and caused cell death in 3T6. This effect is not seen in HeLa or IMR-90 cultures infected with this strain.

The effects of MI on overall growth of various cell cultures have been well documented. Fogh *et al.* (1971) showed that *M. fermentans* reduced the growth rate of FL amnion cells. The population doubling times for the infected and noninfected lines were 30 and 17.5 hr, respectively. Joncas *et al.* (1969) reported

on the effects of *M. hyorhinis* in a human fetal diploid cell line and in a monkey kidney cell line. Effects on growth were not listed, but cytopathology did occur. Sasaki *et al.* (1981) showed that *M. hominis* reduced the growth rate of HAIN-55 human diploid fibroblasts in short-term experiments. The effect depended upon the number of organisms inoculated. Growth inhibition of the host cell culture was also produced with lysates as well as with cytoplasmic and membrane fractions of *M. hominis*. In related studies, Kihara *et al.* (1981) studied infection of *M. hominis* and *M. orale* in HAIN-55 cells, showing that trypsinization and EDTA released mycoplasmas from the host cells. The authors suggested that proteinaceous material and salt bridges might be responsible for mycoplasmal attachment to HAIN-55 cells. Sethi and Brandis (1970) showed that Ehrlich ascites tumor cells adsorbed to colonies of *M. pneumonaie* and *M. gallisepticum*. The adsorption was destroyed when the ascites cells were pretreated with receptor-destroying enzyme. Sialic acid is present in membranes of Ehrlich ascites cells. The same authors (Sethi and Brandis, 1971) reported *in vivo* inhibition of tumor production in mice injected with mycoplasma-treated Ehrlich tumor cells. In the same study, intratumoral inoculation of *M. gallisepticum* into 8- and 17-day-old tumors led to tumor destruction in NMRI mice. The mechanism of this action was not determined, but natural killer cells could be involved (Beck *et al.*, 1980; Birke *et al.*, 1961). Pijoan (1975) reported on primary cultures of pig kidney inoculated with *M. hyorhinis* or *M. hyopneumoniae*. Both organisms multiplied in the culture, although only *M. hyorhinis* produced cytopathology.

B. Effects on Viruses

A broad literature exists on the effects of MI on viral propagation and cell–virus–mycoplasma interactions. These have been reviewed and updated (Barile and Grabowski, 1978; Barile, 1979). Table IV summarizes some data on the effect of MI on viruses. In the study by Singer *et al.* (1969a), infection of hamster embryo cells by *M. arginini* alone had no effect on Semliki Forest virus (SFV), while *M. hyorhinis* actually increased to a slight extent yield of SFV, an RNA virus. Infection of the same hamster embryo cultures by *M. arginini* increased the yield of DNA-containing vesicular stomatitis virus (VSV). The mechanism proposed for increased yield of VSV is mycoplasmal inhibition of interferon which, in turn, increased viral growth. *Mycoplasma arginini* also inhibited interferon induction by synthetic copolymers (Singer *et al.*, 1969b).

Manischewitz *et al.* (1975) showed that *M. arginini* reduced the titers of herpes simplex virus by 10^2–10^3 PFU/ml in the cell culture. The authors suggested that the effect may be due to depletion of arginine since the effect was reversed by addition of excess arginine. *Mycoplasma hyorhinis*, a non-arginine utilizer, had no effect on herpes simplex virus growth. The authors suggested that the arginine depletion effect of mycoplasmas may be a useful probe to

TABLE IV. Some Effects Mycoplasmas Have on Virus Propagation in Cell Cultures

Species	Virus	Cell culture	Effect	Reference
M. arginini	VSV[a]	Hamster embryo	Increased virus yield	Singer *et al.* (1969a)
M. arginini	SFV[b]	Hamster embryo	No significant change	Singer *et al.* (1969a)
M. hyorhinis	SFV	Hamster embryo	Increased virus yield	Singer *et al.* (1969a)
M. orale	Vaccinia	HeLa	Increased virus yield	Hargreaves and Leach (1970)
M. orale	Adeno 2	HeLa	Decreased virus yield	Hargreaves and Leach (1970)
M. orale	Canine distemper	Vero	Decreased virus yield	Hirayama *et al.* (1981)
M. orale	Herpes simplex	HEp-2	Decreased virus yield	Melnick *et al.* (1980)
M. pneumoniae	Influenza A	Chorioallantoic membrane	Decreased virus yield	Nakamura and Sakamoto (1969)
M. arginini	Varicella	MRC-5	Decreased virus yield	Stack and Taylor-Robinson (1973)
Unidentified	SV40, adeno	African green monkey kidney	Decreased virus-DNA labeling	Van Roy and Fiers (1977)
M. arginini	Herpes simplex	Vero	Decreased virus yield	Manischewitz *et al.* (1975)
M. orale	RSV[c]	WI-26	Decreased virus yield	Somerson and Cook (1965)
	RAV[d]	WI-38	Decreased virus yield	Somerson and Cook (1965)
	Adeno type 2	HeLa	Decreased virus yield	Hargreaves and Leach (1970)
	Vaccinia	HeLa	Slight increase virus yield	Hargreaves and Leach (1970)
M. hominis	Adeno type 2	HeLa	Decreased virus yield	Hargreaves and Leach (1970)
	Herpes simplex	HeLa	Decreased virus yield	Hargreaves and Leach (1970)
	RSV	HeLa	Increased virus yield	Hargreaves and Leach (1970)
	Vaccinia	HeLa	Increased virus yield	Hargreaves and Leach (1970)
M. hyorhinis	Adeno type 2	HeLa	Decreased virus yield	Hargreaves and Leach (1970)
	RSV	HeLa	Increased virus yield	Hargreaves and Leach (1970)
	Vaccinia	HeLa	Slight increase virus yield	Hargreaves and Leach (1970)

[a]VSV = Vesicular stomatitis virus.
[b]SFV = Semliki Forest virus.
[c]RSV = Rous sarcoma virus.
[d]RAV = Rous associated virus.

determine the arginine requirement for other DNA viruses. These suggestions confirm the findings of Rouse *et al.* (1963), who showed that arginine-utilizing mycoplasmas inhibited plaque formation of adenoviruses. Goldblum *et al.* (1968) documented the effect of arginine and other amino acid depletions on the synthesis of tumor and viral antigens of SV40. However, Meloni *et al.* (1980) point out that arginine depletion is not the only explanation for decreased viral growth.

Mycoplasmas can affect viruses in other ways. Dickson and colleagues (1980) published an interesting study on the ability of mycoplasmas to abrogate antibody-mediated neutralization of VSV. *Mycoplasma orale*, *M. arginini*, and *M. hyorhinis* all reactivated VSV titers from less than 1×10^1 PFU/ml to $10^{6.7}$, $10^{3.7}$, and $10^{4.7}$, respectively. These findings have important implications in recovery and identification of viruses in cell culture systems. The authors suggest that the reactivation effect may be due to proteases or glycosidases excreted or located on the surfaces of mycoplasmas. Reactivation did not occur with supernatants of mycoplasma cultures. A precedent for this is known: *Tetrahymena pyriformis* excretes a protease that degrades antibodies (Eisen and Tallan, 1977).

C. Tumor Viruses

Van Roy and Fiers (1977) showed that infection of African green monkey kidney cell cultures with an unidentified mycoplasma had no effect on growth of the cell culture, SV40, or adenovirus. Since it had no effect on growth of these DNA viruses, it could be supposed that the mycoplasmas was a non-arginine utilizer. The Van Roy and Fiers study did show that MI drastically reduced the radioactive labeling of viral DNA when nucleosides were used as radioactive precursors, probably due to the action of mycoplasmal nucleoside phosphorylases. It is interesting that a significant reduction of incorporated label occurred even though no effect on the overall viral titers was noted.

Effects on other tumor virus systems have been reported. Fogh and colleagues (1970, 1971) have published several papers on the effects of MI on SV40 transformation of human amnion cells. SV40-transformed cells were more susceptible to MI; transformed cells also had more cell-associated mycoplasmas (Fogh, 1970). It was not known if the unidentified mycoplasma associated with SV40 antigen on the transformed amnion cell membrane. Somerson and Cook (1965) showed that *M. orale* suppressed growth of Rous sarcoma virus.

McPherson and Russell (1966) have reported on the effects of MI on transformation of cell cultures with and without tumor viruses. Mycoplasma-mediated inhibition of Rous sarcoma virus growth decreased cell transformation by this virus. In virus-free systems, the authors showed that *M. fermentans* (PG-18) and *M. hominis* (PG-21 and PG-27) produced effects in BHK21-C13 cells that mimicked transformation by growth in soft agar. Mycoplasma-infected cells had

altered morphologies and grew in soft agar; the clonal efficiencies of these cells ranged from 0.5 to 5.0%. Cells derived from colonies in soft agar retained their altered epitheloid/fibroblastic morphology when grown on glass. Of particular interest was the fact that cultures cured of mycoplasmas by antibiotic treatments retained their high plating efficiencies, 10–150 times higher than those of controls. This indicates that continued MI is not required for the continued observation of the morphological changes. McPherson and Russell (1966) believed the changes they observed were analogous to the irreversible chromosomal changes reported by Fogh and Fogh (1965).

D. Cytogenetic Effects

The cytogenetic effects of MI have recently been reviewed (McGarrity *et al.*, 1984a). Nichols (1978) has also published a brief review of cytogenetic effects of cell culture mycoplasmas.

Fogh and Fogh (1965) published the first report that MI caused chromosomal aberrations. They showed that an unidentified mycoplasma produced a decreased chromosome number in FL amnion cells. This coincided with an increase in open breaks and stable and unstable rearrangements. Antibiotic treatment to eliminate the mycoplasmas resulted in elimination of unstable rearrangements. However, reduced chromosome number and stable rearrangements persisted, indicating that reversible and irreversible changes occurred as a consequence of MI.

In human diploid fibroblasts, *M. orale* produced a three- to five-fold increase in chromosome breaks and rearrangements and number of polyploid cells (Paton *et al.*, 1965). Aula and Nichols (1967) demonstrated that arginine depletion by *M. salivarium* was the mechanism responsible for the threefold increase (5.6 to 18%) in chromosomal abnormalities in human leukocytes. *Mycoplasma hominis* type 2 and *M. fermentans* had no effect in these studies. Addition of 2 m*M* arginine prevented chromosomal damage. In a later study, *M. salivarium* failed to produce an increase in chromosomal aberrations in human lymphocyte cultures (McGarrity *et al.*, 1980a). Arginine concentrations in medium differed in this and the study reported by Aula and Nichols (200 and 126.4 mg/liter, respectively), but this was not thought to be significant.

Mechanisms other than arginine depletion can produce chromosomal aberrations. This has been shown in several studies using non-arginine-utilizing species of mycoplasmas. Kundsin *et al.* (1971) documented that primary isolates of *Ureaplasma urealyticum* produced chromosomal abnormalities in human lymphocytes. This ability was strain specific since only one of two strains tested produced aberrations. Ureaplasmas do not contain arginine deiminase. Standbridge *et al.* (1969) demonstrated that *M. orale*, *M. fermentans*, *M. hominis*, and *A. laidlawii* induced chromosome aberrations in WI-38 fibroblasts. All these organisms except *A. laidlawii* utilize arginine. These workers suggested that

mycoplasmal inhibition of mammalian DNA synthesis was the cause of the so-called leopard nuclei, clumps of chromatin within the nucleus. They noted that the leopard nuclei resembled those seen in cells treated with arabinosylcytosine, an inhibitor of DNA synthesis as described by Nichols and Heneen (1964). The specific effects of arginine depletion were not investigated in this study. Inhibition of DNA synthesis and chromosome breaks could be due to several factors, including arginine depletion.

Other studies showing MI to have no detectable effect on chromosomes have been reported. Aside from the above citation concerning *M. salivarium* by McGarrity *et al.* (1980a), Fogh and Fogh (1973) showed no unusual banding patterns in FL cells infected with *M. fermentans*. More recently we reported no significant increase in chromosomal aberrations in the rabbit eye lens line AG-4676 infected with *Spiroplasma mirum* (Megraud *et al.*, 1983).

Several other reports on mycoplasma-induced chromosomal aberrations have been published, but the mycoplasmal species were not identified. Kuzmina (1972), working with mouse cell cultures, stated that mycoplasmas were the presumed cause of Robertsonian fusion. Schneider *et al.* (1974b), working with human amnion cells used for amniocentesis assays, reported a significant increase in chromosome gaps and aneuploidy in mycoplasma-infected amniotic fluid cells. These workers stressed the potential of false positives in amniocentesis assays. Amniocentesis assays have varying legal implications in different geographic areas. If MI does occur there may not be sufficient time for a repeat sample to be completed and still have a legal abortion, if desired.

E. Effects on Mutagenic Assays

A large number of short-term mutagenicity procedures have been developed. Most of these use *in vitro* systems, especially cell cultures. More than 100 have been developed. The relative advantages and disadvantages of various systems have been reviewed (Hollstein *et al.*, 1979).

Mycoplasmas can have drastic effects on mutagenic assays, leading to qualitatively and quantitatively false results. These effects are due to overall mycoplasmal effects on growth, chromosomes, and possession of metabolically active isoenzymes that can confuse assay results. Table V lists some published and unpublished effects of MI on mutagenic assays. These effects have been reviewed in detail (McGarrity *et al.*, 1984a). The effects on chromosomes have been noted above. In addition, MI can affect related assays, such as sister chromatid exchange (SCE), in two possible ways. First, since mycoplasmas produce chromosomal aberrations, they could theoretically increase SCE frequency. Also, addition of bromodeoxyuridine (BUdR) is a first step in SCE assays. Mycoplasmal uridine phosphorylases cleave BUdR, rendering it unavailable to the cultured cells. This would result in inhibition of chromatid differentiation, requiring higher concentrations of BUdR to be used. The overall effects of MI in this assay are speculative. In most of our unpublished studies we could find little or no effect of MI on SCE.

TABLE V. **Effects of Cell Culture Mycoplasmas on Mutagenic Assays**

Assay	Species	Effect	Reference
Metaphase–anaphase plates	Many	Breaks, gaps, aneuploidy	Many [See McGarrity *et al.* (1984a) for review]
Sister chromatid exchange	Many	Phosphorlytic cleavage of BUdR, requiring higher concentrations of BUdR	McGarrity (unpublished)
DNA repair	*A. laidlawii*	Dark repair	Das *et al.* (1978)
	M. buccale	Dark repair	Aoki *et al.* (1979)
	M. orale	Lack of dark repair	Mills *et al.* (1977)
	M. hyorhinis	Lack of dark repair	
	M. hyorhinis	Inhibition of hydroxyurea of DNA synthesis	Gruneisen *et al.* (1975)
Hypoxanthine-guanine phosphoriboryltransferase	*M. hyorhinis*	HPRT activities, effect in HAT medium, substrate binding	Van Diggelen *et al.* (1978)
	A. laidlawii		
	M. orale		
	M. arginini		
	M. salivarium		
Thymidine kinase locus assays	Unidentified	Interferes with forward and backward mutations	Clive *et al.* (1973)

MI can interfere with DNA repair studies in several ways, depending on the infecting species. However, as Das *et al.* (1978) point out, some of the results of studies on mycoplasmal DNA repair could have been influenced by the methods employed. In an interesting study, Gruneisen *et al.* (1975) document that mycoplasmal DNA responds to hydroxyurea in a manner typical of prokaryotic DNA. Hydroxyurea inhibition of DNA synthesis in prokaryotes is less than that in eukaryotes. In four different cell lines infected with *M. hyorhinis*, additions of 10^{-2} *M* to 10^{-5} *M* hydroxyurea did not inhibit incorporation of tritiated thymidine, whereas incorporation was completely inhibited in mycoplasma-free cells with hydroxyurea. Rosenkranz *et al.* (1967) showed that the concentrations of hydroxyurea inhibiting DNA synthesis in bacteria are two logs higher than those required for a similar inhibition in mammalian cells. Later *et al.* (1975) showed *in vivo* correlates of mycoplasma inhibition of DNA repair. *Mycoplasma arthritidis* strain ISR inhibited DNA repair of splenic cells in Sprague–Dawley rats. This system used hydroxyurea.

Cell culture mycoplasmas can also serve as metabolic sinks for cell culture supplements or mutagenic chemicals, thus reducing the effective concentration of these agents available to the cell culture in the assay. For example, Van Diggelen *et al.* (1978) showed that mycoplasmal HPRTs had greater affinities for 6-thioguanine than the mammalian isoenzymes tested. Mycoplasma-infected HPRT-positive cell lines could result in survival of cells in a mutagenic assay due to mycoplasma metabolism of 6-thioguanine, yielding false positive results. Van Diggelen *et al.* (1977a) reported that mycoplasma-infected cultures do not grow in HAT medium. This extended an early finding of Hakala *et al.* (1963), who showed that mycoplasma-infected HeLa sublines did not propagate in a medium supplemented with amethopterin and thymidine.

Since mycoplasmas possess thymidine kinase (TK), as shown by O'Brien *et al.* (1981), they can influence mutagenic assays at the *TK* locus. Clive *et al.* 1973) have reported on this. Their assay used mouse lymphoma cells heterologous at the *TK* locus (*TK* +/−), enabling the assay to be used for forward or backward mutations. For forward mutations, the cells are grown in the presence of BUdR after addition of mutagens. Cells mutated at the *TK* locus (*TK* + → −) do not incorporate BUdR and are resistant; nonmutated cells incorporate BUdR and are killed. In MI, the cells do not incorporate BUdR, rather the cells act like mutants and survive.

In a variation of the assay, *TK* −/− cells can be treated with a mutagen and selection made for back mutations to *TK* +/− by growing cells in HAT medium. As discussed above, mycoplasma-infected cells will die in HAT medium, yielding false negatives in this assay for backward mutation.

F. Effects on Nucleic Acid Metabolism

Beginning in the mid-1960s, reports began to appear on the effect of MI on nucleic acid metabolism of their host cells. One of the first reports was by Randall *et al.* (1965), using HeLa cells and L cells and an unspeciated mycoplasma. Radiolabeling with [^{14}C]thymidine was performed with mycoplasma-infected and mycoplasma-free cultures. In these experiments the amount of specific activity of DNA did not significantly differ between infected and noninfected cultures, possibly due to the fact that mycoplasmas did not strongly adhere to the monolayer used for DNA extraction. However, radioactivity in the supernatant medium showed drastic differences. In two experiments with L-cell cultures, the counts/min/10 ml were 8100 and 6350 for the mycoplasma-free and 137,000 and 60,900 for the mycoplasma-infected cultures.

Russell (1966) showed in studies with *M. pulmonis*- and *M. fermentans*-infected BHK21-C13 cells that mycoplasmas apparently had thymidine kinase, DNase, RNase, and thymidine phosphorylase activities. He showed that inhibition of uptake of labeled nucleosides may be at least partially due to degradation of substrates by mycoplasmal phosphorylases.

In related studies, Perez *et al.* (1972) showed that altered incorporations of nucleic acid precursors in mycoplasma-infected cells were due to enzymatic degradation of nucleosides by the mycoplasmas. These workers also demonstrated that mycoplasmas could use nucleosides directly. In a thorough study, Hellung-Larsen and Fredericksen (1976) followed the labeling of precursors into RNA components of *M. hyorhinis*-infected cell cultures. [^{32}P]Orthophosphate or [^{3}H]methyl groups were found only in mycoplasma tRNA, 23 S, and 16 S rRNA. [5-^{3}H]Uracil was incorporated into mycoplasmal tRNA, 4.75 S RNA, a mycoplasmal low molecular weight component, M, 23S, and 16 S rRNA. [8-^{3}H]Guanosine or [5-^{3}H]uridine labels were incorporated into host 28 S and 18 S rRNA, but to a significantly lower level than into mycoplasmal 23 S and 16 S rRNA.

McIvor and Kenny (1978) studied representatives of the eight major serological groups of mycoplasmas for their incorporation of free bases and nucleosides. Broth-propagated *A. laidlawii*, *M. hyorhinis*, and *M. arginini* were included. All organisms tested incorporated uracil, adenine, and guanine; none incorporated cytosine. Species frequently isolated from cell cultures did not independently incorporate thymine, but deoxynucleosides enhanced thymine incorporation in *A. laidlawii* and *M. hyorhinis*. No significant nucleoside incorporation occurred in the arginine-utilizing species, an important finding if it is true of all arginine utilizers. Significant differences in incorporation of bases and nucleosides were detected in the species tested. Strain differences are also likely. This and other work demonstrate the potential of MI to invalidate results of studies on mammalian transport and incorporation of nucleosides and free bases. It also demonstrates potential risks associated with using incorporation of radiolabels as an assay for MI since species and strain differences do occur.

All mycoplasmas tested in one study lacked the orotic acid pathway for pyrimidine synthesis and the enzymatic pathways for *de novo* synthesis of purine, so that at least one pyrimidine and one purine base must be supplied by the cell culture. Mycoplasmas incorporate free bases and nucleosides with equal effectiveness. On the other hand, mammalian cells in culture incorporate free bases to a negligible level only. Plageman and Richey (1974) showed this to be due to the lack of phosphoribosyltransferase activity and not to lack of transport of free bases.

G. Effects on Carbohydrate Metabolism

Since two of the four mycoplasma species isolated from cell cultures are classified as fermenters, they can be expected to significantly influence the fermentative pathways of their host cells. Surprisingly little direct data are available in this area. Many more studies have been published on the "arginine effects" of MI than on the fermentive effects. As mentioned by Stanbridge and Doerson (1978), "The deleterious effect of fermentative mycoplasmas has been

ascribed to adverse acid pH conditions and to competitive utilization of nucleic acid precursors. . . ." While the area of nucleic acid precursors has been vigorously studied, relatively few studies have been published on specific effects on carbohydrate metabolism in infected cell cultures.

Butler and Leach (1964) reported on cytotoxic effects of a fermentative mycoplasma in cell cultures, possibly as a result of acid pH. Williams *et al.* (1981) reported that mycoplasma-infected human KB cells exhibited significantly higher glycolytic and respiratory rates than mycoplasma-free cells. Unfortunately, the mycoplasma was not identified. Respiration was the most affected parameter in this study. The micromolar uptake of oxygen per hour was 47–126% higher in infected cells and production of CO_2 was 31–145% higher. Approximately 69–145% more glucose was utilized by infected KB cells. No difference was noted in lactate production. These authors suggested a possible independent hexose monophosphate shunt activity in mycoplasmas.

Clark *et al.* (1978) reported increases in pyruvate dehydrogenase activity (PDH) in *M. pneumoniae*-infected human fibroblasts. We have extended these studies to show that fermentative mycoplasmas *A. laidlawii* and *M. hyorhinis* had PDH and PDH complex activity while nonfermenters *M. arginini*, *M. orale*, and *M. salivarium* did not (McGarrity *et al.*, 1984c).

H. Effects on Lymphoblastoid Cells in Culture

The exciting proliferation of *in vitro* studies on lymphoblastoid cells and their mechanisms of action in recent years has been accompanied by an increased number of reports on the effects of mycoplasmas on such systems. Among other things, many of these reports have generated useful information on immunological responses to mycoplasmas *in vitro*. We have reviewed earlier studies in this field, including effects on lymphocyte stimulation, interferon induction, and chromosome damage (McGarrity *et al.*, 1978; see Chapter 6, this volume).

Ginsburg and Nicolet (1973) reported that *M. pulmonis* stimulated rat lymphocytes at a high efficiency, 85% blast cells being detected within 4 days. They also demonstrated the lack of specificity of this reaction since lymphocytes from rats free of *M. pulmonis* and of circulating anti-*M. pulmonis* antibody responded in a manner similar to *M. pulmonis*-infected animals. Fernald (1972) reported that human lymphocytes from individuals with circulating antibodies to *M. pneumoniae* were stimulated with this organism to a level comparable to phytohemagglutinin (PHA). Spitler *et al.* (1968) first showed that mycoplasmas inhibited PHA stimulation of lymphocytes. This system used *M. arthritidis* strain PN and goat lymphocytes. Addition of the organisms as late as 45 hr after PHA stimulation prevented RNA and DNA synthesis. The mechanism of action was not due to killing of the cells, a combination of mycoplasmas and PHA, or competition for cell receptors. In a study of five mycoplasma species, Barile and Leventhal

(1968) demonstrated a correlation between lymphocyte stimulation and the fermentative mycoplasmas *M. pulmonis* and *M. pneumoniae*. These authors showed that the arginine-utilizing mycoplasmas *M. hominis*, *M. orale*, and *M. arthritidis* inhibited PHA activity. Inhibition could be reversed with excess arginine. In the same year, Cole *et al.* (1977) demonstrated that nonviable preparations of fermentative mycoplasmas were mitogenic for mouse lymphocytes. Mycoplasma species shown to be active included *A. laidlawii*, *Spiroplasma citri*, *M. synoviae*, *M. gallisepticum*, *M. pneumoniae*, and *M. fermentans*. These *in vitro* studies suggested that the ability of mycoplasmas to activate normal lymphocytes provided the potential for an early inflammatory response before the development of a sensitized lymphocyte population. The reaction of persistent mycoplasma antigens with sensitized lymphocytes would produce continued inflammation. The inhibition of PHA stimulation of lymphocytes by arginine-utilizing mycoplasmas raises the question of whether such a mechanism might exist *in vivo*. As Spitler *et al.* pointed out in 1968, depressed response to PHA has been noted in diseases associated with a defect in immune response and in certain viral diseases.

Recently, Jakway and Shevack (1984) reported another practical aspect of inhibition of lymphocyte stimulation by mycoplasmas, namely, as a screen for monoclonal antibodies to soluble mediators of immune responses and to receptors for these mediators.

Cole and colleagues (1977) have greatly contributed to understanding the interactions between mycoplasmas and lymphocytes. In the above-cited study, they suggested that the mycoplasmal factors responsible for stimulation of lymphocytes were heat labile and independent of endotoxic activity. Use of *M. arthritidis* in a model system for lymphocyte cytotoxicity tests showed that lymphocytes preincubated with mycoplasmas were toxic to a variety of target cells, including fibroblasts (Cole *et al.*, 1980). Lymphocyte populations devoid of B cells did not affect killing of target cells. In a later study, Cole *et al.* (1981) demonstrated that the induction of cytotoxic lymphocytes was under the control of the *Ir* gene locus (see Chapter 6, this volume).

Standbridge and Weiss (1978) reported infection by *M. hyorhinis* and subsequent patching and capping of the mycoplasmas on mouse lymphocyte cells. The caps seemed to be shed from the surface of the cells; reinfection of the stripped cells did not occur. These authors suggested that capping may have a role in mycoplasma pathogenesis, although specific mycoplasmal receptors were not isolated or identified. More recently Butler and Stanbridge (1983) identified the receptors.

Wise *et al.* (1978), in a study of *M. hyorhinis* infection of mouse lymphocytes, showed that *M. hyorhinis* could selectively strip Thy-1.1 differentiation alloantigens and H-2K^k histocompatibility antigens from the lymphocyte surface. This study was performed in a murine T lymphoblastoid line, BW 5147. The Thy-1

was recovered from *M. hyorhinis* membranes. On the other hand, the gp70 molecule, also present on the cell surface, was not detected in the *M. hyorhinis* membrane, suggesting some selectivity. These observations have been updated (Wise *et al.*, 1979).

In an interesting extension of these studies, Tarshis *et al.* (1981) studied the interactions of *A. laidlawii* and murine splenic lymphocytes. Membrane uncouplers and chelators (EDTA) did not influence mycoplasmal attachment, and neither did glutaraldehyde nor neuraminidase pretreatment of the lymphocytes. These results suggested that protein receptors and sialic acid receptors are not involved in attachment of *A. laidlawii* to murine lymphocytes. Nor is pinocytosis. The results of these studies suggested that attachment of *A. laidlawii* to splenic lymphocytes is followed by fusion of the two cells. Unsaturated fatty acids such as [^{3}H]oleic acid were transferred from the mycoplasmas into the lymphocyte membranes. Conversely, incubation of *A. laidlawii* with lymphocytes led to an increase of cholesterol in the mycoplasmas of up to 49 μg/mg protein while the cholesterol content in lymphocytes decreased from 260 to 180 μg/mg protein. Membrane fusion could also result in intermembrane lipid and protein exchange, as observed by Wise *et al.* (1978). Grant and McConnell (1973) reported that *A. laidlawii* cells fused with lipid vesicles. In related studies, Gabridge *et al.* (1977) suggested that *M. pneumoniae* fused with cells of tracheal organ cultures without the participation of receptors. Stanbridge and Weiss (1978) reported on mycoplasma capping in lymphocytes, and Butler and Stanbridge (1983) on the identification of the receptors.

In this section on lymphoblastoid cells, special mention should be made regarding monoclonal antibody-secreting hybridoma cells. Serious difficulty has been encountered with respect to MI. This is probably due to the high incidence of MI in parental myeloma cultures. We have noted a high incidence, as high as 40%, in myelomas from different laboratories. In fact, we know of two instances where monoclonal antibodies had been inadvertently and unknowingly prepared against mycoplasmas that were present as contaminants in preparations obtained from mycoplasma-infected cultures. Vennegoor *et al.* (1983) reported on a similar case in which monoclonal antibodies were inadvertently prepared against *M. hyorhinis* in this manner. Buck *et al.* (1982) produced monoclonal antibodies to broth-propagated mycoplasma species routinely isolated from cell cultures.

I. Effects on Interferon

As mentioned earlier, some effects of MI on viral propagation and viral titers can be mediated through interferon (IFN). In different studies, mycoplasmas have been shown to directly induce IFN or to inhibit IFN induction. Singer *et al.* (1969a) were the first to suggest that MI could increase viral yields through decreased IFN induced by Semliki Forest virus and by polyinosinic and polycytidilic acid. Only one of the species studied utilized arginine, suggesting that mechanisms other than arginine utilization can be involved. Neither mycoplasmal organism induced IFN in this study. In a subsequent report, Singer and Ford (1972) showed that the effects of *M. arginini* and *M. hyorhinis* on reduced IFN titers achieved with poly (I)·poly(C) were reversed by DEAE-dextran but not by neomycin. Cole *et al.* (1975) confirmed this finding with poly (I)–poly(C) *in vivo* with mice using *M. arthritidis*.

Rinaldo *et al.* (1974a) showed that twelve different *Mycoplasma* and *Acholeplasma* species induced IFN in ovine peripheral blood leukocyte cultures. These included several species that are isolated from cell cultures: *A. laidlawii*, *M. orale*, *M. fermentans*, *M. hyorhinis*, *M. buccale* (reported as *M. orale* type 2), *A. granularum*, *M. salivarium*, and *M. hominis*. Two other species isolated from cell cultures, *M. arginini* and *A. axanthum*, did not induce IFN. Extending this investigation to *in vivo* studies on Swiss Webster mice, Rinaldo *et al.* (1974b) showed that three mycoplasma species, *A. laidlawii*, *M. pneumoniae*, and *M. arthritidis*, induced IFN; *M. pulmonis* did not. IFN was not induced by these mycoplasmas in mouse peripheral blood leukocytes or in spleen and peritoneal cells.

Cole *et al.* (1976) demonstrated the *M. pneumoniae*, *A. laidlawii*, *M. arthritidis*, and *M. pulmonis* induce IFN in the lymphocyte fraction of ovine peripheral blood leukocytes; polymorphonuclear leukocytes were not involved. Human peripheral blood lymphocytes were stimulated to produce high levels of IFN, up to 230 units by *M. synoviae*, and lesser levels with *M. pneumoniae*. *Acholeplasma laidlawii*, *M. hominis*, *M. orale*, *M. buccale*, *M. faucium*, *M. fermentans*, *M. salivarium*, and *M. gallisepticum* failed to induce IFN in this system. The authors point out that there did not appear to be a correlation between the ability of mycoplasmas to adsorb to mammalian cells and their ability to induce IFN in sheep leukocyte cultures. On the other hand, *M. pneumoniae* and *M. synoviae*, inducers of IFN in human leukocytes, have a high avidity for various cells.

More recently, as more immunologists begin to utilize cell cultures as research tools, papers have appeared on the effects of MI on parameters of cellular immunology. Two interesting papers are those of Beck *et al.* (1980) and Birke *et al.* (1981). In the first paper, Beck *et al.* reported that all lymphoma cells in a murine system that induced an acid-labile IFN in the mixed lymphocyte tumor cell assay were infected with mycoplasmas. Further, cellfree supernatants of mycoplasma-infected tumor cell cultures induced IFN. The isolate was not identified. As part of this study, these workers showed that *A. laidlawii* and *M. pneumoniae* induced 30 and 64 units IFN/ml, respectively, in spleen cell cultures of C57/BL6 mice.

Birke *et al.* (1981) performed similar studies with human tumor cells. Four melanoma lines and one ovarian carcinoma line infected with *M. orale* induced

heat- and acid-stable human leukocyte interferon, α-IFN. α-IFN was not induced in these lines when mycoplasmas were eliminated by mouse macrophage treatment. Their studies also showed that *M. orale*-infected lines were more susceptible to natural killer (NK) cell-mediated lysis than were mycoplasma-free cultures. These workers could not unequivocally conclude that NK lysis was due solely to MI of target cells, since some lysis occurred in the absence of mycoplasmas. Cellfree supernatants did not induce IFN in human leukocytes. These findings extend the spectrum of human NK target cells from tumor- and viral-infected cells to cells infected with mycoplasma. In fact, Birke *et al.* speculate that this phenomenon may operate *in vivo*.

In related studies, Loewenstein *et al.* (1983) reported that MI with *M. orale* induced macrophage-mediated cytolysis of mouse A9 tumor cells. Both syngeneic and allogeneic peritoneal macrophages were effective. A ratio of 100 CFU *M. orale* per mouse macrophage produced 64% cytolysis in A9 cells and 1% in normal mouse fibroblasts. Similar results were obtained with heat-killed *M. orale*, which would argue against metabolic effects such as arginine depletion of the medium. Lymphocyte involvement was also ruled out in this study. Direct activation of the macrophage was suggested. Similar results were obtained when macrophages were obtained from untreated, thioglycolate-treated, or axenic mice. The degree of killing was correlated with the number of *M. orale*. These workers also suggested that MI might account for at least some of the reports on neoplastic cell lysis by nonactivated macrophages. Weinberg *et al.* (1980) showed that lipoglycans from *A. axanthum* or *A. granularum* did not produce *Limulus* lysate clotting or tumor cell killing by mouse peritoneal macrophages.

J. Isoenzyme Studies

The effects of MI on some enzyme systems (TK and HPRT) have been described. In other studies, Bradlaw *et al.* (1982) reported that *M. arginini* infection increased the responsiveness of rat hepatoma H-4-II-E cells to aryl hydrocarbon hydroxylase (AHH) induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Elimination of the infection by antibiotic treatment rendered the cells less responsive to AHH induction by TCDD. Almost double the TCDD dose was required to achieve half maximal enzyme induction.

In a comprehensive study, O'Brien *et al.* (1981) described isoenzyme expression in 22 *Mycoplasma* and *Acholeplasma* species. A total of 21 isoenzymes were assayed. Most mycoplasmas assayed had significant activity of 9 isoenzymes: triose phosphate isomerase, glucose phosphate isomerase, dipeptidase, glyceraldehyde-3-phosphate dehydrogenase, adenylate kinase, inorganic pyrophosphatase, acid phosphate esterase, and nucleoside phosphorylase. Acholeplasmas, but not mycoplasmas, contained superoxide dismutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. The detection of the latter two enzymes indicates that the pentose shunt may be functional in acholeplasmas but not in mycoplasmas. The expression of mammalian isoenzymes was not apparently altered by mycoplasmas. On the basis of these studies, triose phosphate isomerase, glucose phosphate isomerase, and nucleoside phosphorylase are the best isoenzymes to monitor for MI. However, such a monitor can be significantly influenced by the level of activity in the infecting mycoplasma and the coincidental mobilities of homologous enzymes produced by the host cells and the infecting mycoplasmas.

Clark *et al.* (1978) reported that mycoplasmas have higher relative activities of pyruvate dehydrogenase (PDH) and pyruvate dehydrogenase complex (PDHC) relative to human fibroblasts. Constantopoulos *et al.* (1981) measured several oxidative enzymes in fermentative and nonfermentative mycoplasmas. They found high activities of PDH and PDHC in the fermentative *A. laidlawii*, *M. hyorhinis*, and *M. pneumoniae*. Activity of α-ketoglutarate was minimal in all five mycoplasmas tested. *Acholeplasma laidlawii* had lipoamide dehydrogenase activity comparable to that of human fibroblasts, and all five mycoplasmas examined contained lactate dehydrogenase and NADH-DCPIP oxidoreductose activities. Increased levels of PDH were detected in a human cell line deficient in PDH activity after MI (McGarrity *et al.*, 1984c).

K. Specialized Cell Culture Systems

As more epithelial and other differentiated cells are successfully maintained *in vitro*, the spectrum of MI may be altered to reflect the interactions of mycoplasmas with these cells. What effect will maintenance of cells in serum-free media have on MI? Will mycoplasmas be able to survive and grow in cell cultures maintained in media devoid of animal serum and supplemented with growth factors? Will *Mycoplasma* species, as opposed to *Acholeplasma* species, infect serum-free cell cultures with little or no cholesterol present in medium? Some studies in this laboratory indicate that acholeplasmas, but not mycoplasmas, can grow in serum-free cell cultures.

In addition to serum-free cultures, recent studies have documented effects of MI on certain cell parameters for the first time. Hatcher (1983) showed that human diploid fibroblasts infected with *M. hyorhinis* had significantly higher levels of plasminogen activator compared to uninfected controls. Hatcher noted an approximate 10-fold difference within 48 hr. *Mycoplasma orale* has been shown to induce increased levels of collagenase in BALB/c 3T3 mouse fibroblasts; the enzyme is of mammalian origin (Kluve *et al.*, 1983). Kouri *et al.* (1983) demonstrated that *M. pulmonis* had differing effects on protein and glycosaminoglycan synthesis in human synovial cells, fetal skin fibroblasts, and rat granulation tissue fibroblasts. Hyaluronic acid and sulfated glycosaminoglycans were stimulated by *M. pulmonis* when MI decreased cell density. If MI

stimulated cell growth, hyaluronic acid and glycosaminoglycan levels were reduced. In other studies, Kamatani *et al.* (1983) reported that assumed putrescine auxotrophy in cell cultures was, in fact, due to infection with *M. orale*. Putrescine inhibited *M. orale* growth.

L. Spiroplasma and Ureaplasma Infection of Cell Cultures

Spiroplasmas have not been isolated from uninoculated cell cultures. However, Steiner *et al.* (1982) studied *Drosophila* Dm-1 cell cultures for their response to representatives of the major groups of spiroplasmas. Spiroplasmas could propagate and produce cytotoxicity in Dm-1, which varied with the infecting spiroplasma. These findings may have significance in attempts to isolate spiroplasmas from insects and to study mechanisms of pathogenicity and insect vector–spiroplasma relationships.

MI has been documented in insect and other invertebrate cell cultures. Steiner and McGarrity (1983) recently reviewed this field. Some detection methods may not be efficient for detecting MI in invertebrate cultures. The different temperatures of incubation of invertebrate cultures can be a significant influencing factor. Some detection methods may have to be modified in the mycoplasma assay of invertebrate cell cultures. These points are reviewed in Steiner and McGarrity (1983).

Relatively few studies have been performed in cell and organ cultures with *Ureaplasma urealyticum*. *Ureaplasma urealyticum* is not encountered in mycoplasmal infection of cell cultures. We have not isolated this organism from more than 20,000 cell cultures assayed. The only report of a ureaplasma isolation from cell cultures was by Sethi (1972). Several reasons may explain why the organism is not encountered in cell cultures. Optimal pH for *U. urealyticum* is 6.0, below that required for mammalian cell cultures. The organism may not propagate in many cell culture systems. Plating directly onto conventional mycoplasma agar would produce "T" or tiny colonies and could be interpreted as artifacts, especially in the presence of cultured cells and cell clumps. Shepard and Masover (1979) established a 48-hr infection in HeLa-S3 cell culture. Other attempts to infect HeLa, McCoy, and other cell cultures failed. Mazzali and Taylor-Robinson (1971) established short-term ureaplasmal infections in L-132, HeLa, and Vero. Urea in the medium may have facilitated establishment of infection in these cultures. Masover *et al.* (1976) infected WI-38 with *U. urealyticum* and demonstrated cytopathological effects of the infection; however, the authors reported that *U. urealyticum* strain T960 did not hydrolyze urea in cell cultures. Shepard and Masover (1979) have made a similar observation. This observation may have significant implications in the host–parasite relationship *in vitro* and perhaps *in vivo*. More recently, we have been able to infect 3T6, HeLa, and CV-1 cell cultures with a wide variety of human and animal ureaplasmas without supplemental urea. Urea is present in low concentrations in bovine serum, of the order of 6–8 mg percent (Kotani and McGarrity, 1985).

Organ cultures have also been used. Taylor-Robinson and Carney (1974) showed that genital and oral mycoplasmas multiplied in genital organ cultures without apparent cytopathology. Mårdh *et al.* (1976), using the same system, demonstrated ciliary swelling (1976) after *M. hominis* infection. Stalheim *et al.* (1976) also demonstrated ciliary swelling in bovine oviduct cultures after inoculation of bovine ureaplasmas. McGhee *et al.* (1976) have developed a quantitative technique to determine ciliary action. Human and bovine ureaplasmas grew and produced cytopathology in bovine uterine tube organ cultures, producing a complete cessation of ciliary action within 144 hr. Titers of 10^8–10^9 color change units (CCU) per milliliter were reported.

III. METHODS OF DETECTION

A variety of techniques have been developed and proposed to detect cell culture mycoplasmas. These have been reviewed in detail (McGarrity, 1982). Detection methods are also described in Tully and Razin (1983). Regardless of the method used, attention must be paid to quality control procedures to ensure maximum efficiency. Positive and negative controls must always be included. In fact, Low (1974) has reported contamination of serum-free tissue culture medium. Improper handling of cell culture specimens can affect the results of mycoplasmal assays (McGarrity *et al.*, 1979a). More recently, we have isolated *A. laidlawii* from commercially prepared yeast extract (G. J. McGarrity and H. Kotani, unpublished observations).

Various detection methods are described in Table VI. Several studies have been published on the relative efficiency of different detection methods (Hessling

TABLE VI. Methods to Detect Cell Culture Mycoplasmas

Method	Reference
Microbiological culture	McGarrity *et al.* (1979a)
DNA fluorescent staining	Del Giudice and Hopps (1978)
Immunofluorescence	Del Giudice and Hopps (1978)
Uridine phosphorylase	Levine and Becker (1978)
Uridine–uracil ratio	Schneider *et al.* (1974a)
Scanning electron microscopy	Phillips (1978)
Autoradiography	Studzinski *et al.* (1973)
RNA speciation	Todaro *et al.* (1970)
Mycoplasmal-mediated cytotoxicity	McGarrity and Carson (1982)

et al., 1980; McGarrity *et al.*, 1979b; McGarrity and Carson, 1982). Typically, cell cultures should be assayed 3–4 days after passage. Mycoplasmas, if present, grow to large concentrations in the supernatant fluid and are also adsorbed onto the cultured cells by this time. Cell cultures should be passaged in antibiotic-free media for a minimum of two passages. Antibiotics in media can be a major cause of false negatives in all assays (McGarrity *et al.*, 1979a). Monolayers should be removed from the vessel surface by scraping since proteolytic enzymes can kill mycoplasmas. Cell supernatants will contain 10^7–10^8 CFU/ml; additional organisms are adsorbed onto host cells. Most assay systems monitor organisms adsorbed onto cultured cells; fewer monitor supernatant organisms. In fact, the first step in many assays is the discard of supernatants. Cells and tissues should be assayed promptly after collection or frozen. Kaklamanis *et al.* (1969) demonstrated that lysolecithins were mycoplasmacidal substances in normal tissue extracts.

A. Microbiological Medium

The basic medium for cell culture mycoplasmas is the Hayflick modification of the Edwards medium formulation, consisting of mycoplasma broth base, 5% yeast extract, 20% horse serum, and supplements of 0.5% arginine, 0.5% dextrose, and phenol red (Edwards, 1947). For agar medium, the minimum concentration of a washed agar, such as Noble agar (Difco Laboratories, Detroit, MI), to produce a gel is added. This is generally 0.9%. Final pH is 7.2.

Cell culture specimens are inoculated into broth and onto agar in volumes of 0.1 and 0.2 ml, respectively. Both spent medium and scraped cells should be inoculated. Broths are transferred to agar plates after 7 days. Anaerobic incubation is significantly more efficient than aerobic incubation. In a survey of 6095 cell cultures, aerobic incubation detected 48% of mycoplasmas, and anaerobic incubation detected more than 98% (McGarrity *et al.*, 1979a). We have documented the efficiency of the Gas Pak System (BBL, Cockeysville, MD) for anaerobic incubation (McGarrity and Coriell, 1973). The degree of anaerobiosis can be readily monitored with commercially available methylene blue indicators (BBL, Cockeysville, MD). An acidic or alkaline shift in pH does not constitute a presumptive diagnosis since the cell culture inoculum can induce pH shifts.

Inoculated plates are observed under 100× magnification for the presence of mycoplasmal colonies. Plates are kept for 2 weeks before a negative result is recorded. Most isolates, however, grow in 4–7 days. Fried egg colonies are not typically seen on primary isolations. Some confusion can be caused by the presence of cell clumps and "pseudocolonies"; clumps of cultured cells can mimic smaller, developing mycoplasmal colonies. Pseudocolonies are calcium and magnesium soaps of fatty acids that can also mimic true colonies (Hayflick, 1965). While cell clumps and pseudocolonies may confuse inexperienced personnel, they can be distinguished from true colonies by means of the Dienes stain, a mixture of methylene blue, azure blue, maltose, and Na_2CO_3. Mycoplasma colonies develop a blue color subsequent to Dienes staining, while pseudocolonies and cell clumps remain colorless.

Until 1973, it was believed that the above media formulations would detect all cell culture mycoplasmas. In that year Hopps *et al.* (1973) described a strain (DBS 1050) of *M. hyorhinis* that did not propagate on agar. It was subsequently shown that strains that did not grow on conventional mycoplasmal medium represented a significant portion of cell culture isolates of *M. hyorhinis*. Del Giudice and Hopps (1978) reported that 244 of 394 (61.9%) cell culture strains of *M. hyorhinis* failed to grow on agar. They were shown to be *M. hyorhinis* by immunofluorescence. We found that 33 of 41 (80%) *M. hyorhinis* isolates did not grow on agar (McGarrity *et al.*, 1980b). More recently, approximately 80% of our *M. hyorhinis* isolates failed to grow on agar. It is now known that factors in yeast extract inhibit the growth of these strains (Del Giudice *et al.*, 1980). Pospisil *et al.* (1971) reported similar findings with primary isolates of *M. hyorhinis* from swine; more were detected by immunofluorescence with specific antiserum than by culture. Of course, these findings with primary isolates may be influenced by the tendency of *M. hyorhinis* to occur in "patches" of colonization in the lungs.

Stringent quality control procedures are required for media components. New lots of media components should be assayed with a wild-type organism, such as *M. orale* or *M. arginini*, to ensure proper growth promotion before the component is used routinely in media. Prepared media are stored in 4°C. Mycoplasmal broth has a long shelf life, on the order of 3–4 months. Agar plates are used within 2 weeks; this shelf life can be prolonged by wrapping the plates in aluminum foil or in airtight plastic bags.

B. Indirect Detection Methods

Over the years a variety of techniques have been developed to detect gene products common to mycoplasmas or prokaryotes in general, but not to mammalian cells in culture. While these can be of value, the application of such systems should be monitored carefully to ensure that the method will detect all mycoplasmas encountered in cell cultures and will not produce false positive assays. Further caution is advised in the adaptation of certain biochemical detection procedures to various types of differentiated cell cultures. Most of the data regarding MI of cell cultures have come from fibroblast and lymphocyte cultures, including data on indirect detection methods. Such detection methods may not be applicable to differentiated cell systems. For example, uridine phosphorylase activity, present in mycoplasmas and prokaryotes, but absent from mammalian fibroblasts *in vitro*, has been used as an indirect assay method. It has

been shown, however, that endothelial cells in culture express uridine phosphorylase activity (E. M. Levine, personal communication; G. J. McGarrity, unpublished observations).

Some potential problems can be minimized by the use of an appropriate and effective indicator cell culture system. Unknown specimens are inoculated into the indicator cell culture and, after an appropriate incubation period, the mycoplasmal assay is performed on the indicator cell culture. An indicator cell culture, known to be free of mycoplasmas and free of the characteristics to be measured, improves standardization of the assays, allows positive and negative controls to be performed, and facilitates assays of large numbers of specimens. An appropriate indicator culture should be susceptible to the mycoplasmas likely to be encountered, exhibit a minimal background to prevent false positive results, be easily propagated, be readily available, and have an infinite life-span.

We have used 3T6 mouse embryo fibroblasts as indicators for use in DNA staining with Hoechst 33258, in immunofluorescence for *M. hyorhinis* and other mycoplasmas, and in mycoplasma-mediated cytotoxicity with 6 methylpurine deoxyriboside (McGarrity *et al.*, 1979a; McGarrity and Carson, 1982). Del Giudice and Hopps (1978) have used Vero monkey kidney cells. Stocks of the indicator culture should be frozen in liquid nitrogen and fresh stocks introduced periodically, e.g., every 3 months, to minimize the potential of infection or phenotypic change.

C. DNA Staining

A variety of fluorescent dyes that bind specifically to DNA have been used in cytogenetics. Russell *et al.* (1975) and Chen (1977) introduced two dyes, 4′,6-diamidino-2′-phenylindole (DAPI) and Hoechst 33258, respectively, for detection of MI. The rationale behind this assay is that mycoplasma-free cultures exhibit only nuclear fluorescence. Mycoplasma-infected cultures also display extranuclear fluorescence (Fig. 2). Mitochondrial DNA is not apparent in preparations stained with either DAPI or Hoechst 33258.

Del Giudice and Hopps (1978) first used a 3T6 indicator cell in conjunction with Hoechst 33258 staining, although Vero cells are now used in that laboratory. They reported an efficiency of approximately 98% with this procedure, compared to the combination of microbiological culture and immunofluorescence specific for *M. hyorhinis*. This was based on 2297 specimens. We reported similar results, and our current data, based on approximately 14,000 specimens, indicate an efficiency of about 99% (G. J. McGarrity and H. Kotani, unpublished observations). Considering the failure to cultivate significant numbers of *M. hyorhinis* strains on microbiological medium, fluorescent DNA staining of indicator cell cultures is the single most efficient method to detect MI, based on tens of thousands of cell cultures in several laboratories. Our few false negatives

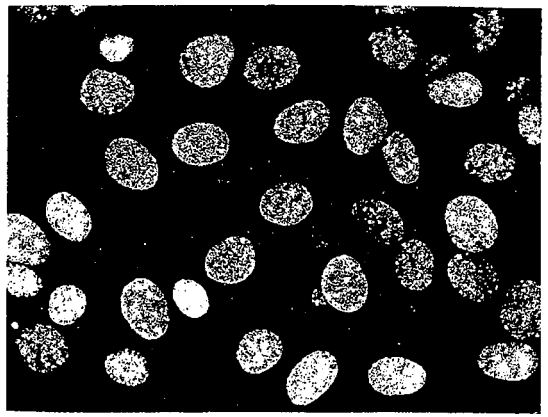

FIGURE 2. HeLa cell culture infected with *M. hyorhinis*, stained with Hoechst 33258.

were strains of *M. arginini* and *M. orale* that grew in cell culture supernatants, but did not adsorb to cell monolayers. Artifacts in this system include fragmented nuclei, which can be confusing to personnel learning the technique.

D. Mycoplasma-Mediated Cytotoxicity

We (McGarrity and Carson, 1982) have developed an indirect detection method using a purine analog, 6-methylpurine deoxyriboside (6MPDR). 6MPDR is nontoxic to mammalian cell cultures. Mycoplasmal adenosine phosphorylase converts 6MPDR into two mammalian antimetabolites, 6-methylpurine and, in the presence of ribose 1-phosphate, 6-methylpurine riboside. These antimetabolites can kill target mammalian cell cultures in concentrations as low as 1 μM. In mycoplasma-infected cultures, 6MPDR completely destroys the cell monolayer in 3–4 days.

In studies using 10 μM 6MPDR and 3T6 indicator cell cultures, 42 of 42 mycoplasma-infected cultures were detected. More recently, we have been performing double-blind prospective studies to determine the efficiency of 6MPDR relative to the combined assays of DNA staining and microbiological culture. To date, 932 cell cultures have been assayed. Of these 34 were infected (3.6%).

There has been complete agreement between 6MPDR assay and the combination of DNA staining and microbiological culture. Controls include the 3T6 indicator culture inoculated with the unknown specimen with and without 6MPDR. If toxicity does occur in the 6MPDR, the control culture without 6MPDR can be used for identification tests.

Possible artifacts include cytotoxicity induced by the infecting mycoplasma in the absence of 6MPDR. The infectious nature of this cytotoxicity could be ascertained through serial dilution and passage to other cell cultures. We have not encountered this in more than 2 years of experience with 6MPDR. Adenosine phosphorylase activity has recently been reported in mycoplasma-free bovine serum (Verhoeff *et al.*, 1983), but should not interfere with 6MPDR at the levels reported. 6MPDR is available commercially (Mycotech, Bethesda Research Products, Gaithersburg, MD).

Mycoplasma-mediated cytotoxicity by 6MPDR is actually a biochemical detection method based on adenosine phosphorylase. The points mentioned below for biochemical detection methods also apply to 6MPDR.

E. Biochemical Detection Methods

Most biochemical detection methods assay for prokaryotic DNA, RNA, or gene products, products that differ from those elaborated by mammalian cells in culture. Too frequently, however, such methods are proposed by cell biologists based on limited studies using few and sometimes unidentified mycoplasmas, and lacking proper controls. Investigators should objectively analyze whether the technique under consideration will detect all mycoplasmas commonly encountered in cell cultures, and be applicable to the type of cell culture of interest.

One of the most widely used biochemical procedures is measurement of uridine phosphorylase (UdR-P) activity. UdR-P activity is not expressed *in vitro* by mammalian fibroblasts, lymphocytes, and certain other differentiated cultures. The assay monitors the conversion of [^{14}C]uridine to [^{14}C]uracil, separation of these by paper chromatography, and subsequent radioactivity counting (Levine, 1974). The technique has been widely used with a high degree of success. However, some mycoplasma-free cultures have been shown to express UdR-P activity, including African green monkey kidney (Van Roy and Fiers, 1977), *Drosophila* 1-XII (Steiner *et al.*, 1982), human endothelial, and some human hepatoma liver cell lines (G. J. McGarrity, unpublished observations).

Hatanaka *et al.* (1975) and Long *et al.* (1977) described the use of adenosine phosphorylase and uracil phosphoribosyltransferase activity as detection methods using radiolabeling. Adenosine phosphorylase activity was detected with this method in all mycoplasmas tested except *M. pneumoniae* FH, *M. pirum*, and *M. lipophilum*. We have detected adenosine phosphorylase activity in all these organisms using 6MPDR.

Uitendaal *et al.* (1979) developed a shortened version of the adenosine phosphorylase assay. Their test measures the conversion of [^{14}C]adenosine performed on plastic leaflets cut from the plastic petri dish in which the cells were grown. This procedure measures the more active reaction in the nucleoside-forming direction. The mycoplasmas used in this study were not identified.

Bonissol *et al.* (1984) used adenosine phosphorylase to screen for mycoplasmas in bovine serum. However, according to their data, 10^6 CCU/ml of *M. orale* are necessary for a positive response. Further, filtered bovine sera had been shown to be mycoplasma-free by isolation technique. In fact, 53 of 138 sera tested had adenosine phosphorylase activity, even though cell culture results were negative. The conclusion of these authors, that the "absence of adenosine phosphorylase activity seems to be the best guarantee that a serum is not contaminated by mycoplasmas," is indeed puzzling. The finding of enzymatic activity by Bonissol *et al.* (1984) may relate to the similar results of Verhoeff *et al.* (1983) and not to contamination with viable organisms.

Nardone *et al.* (1965) first used autoradiography to detect MI, showing cytoplasmic rather than nuclear labeling after incorporation of tritiated thymidine. Studzinski *et al.* (1973) combined nucleoside cleavage and autoradiography to detect MI, claiming that this technique was superior to microbiological culture. However, these workers used aerobic, not anaerobic, incubation in their microbiological procedures. We have shown that aerobic incubation alone detected approximately 48% of infections (McGarrity *et al.*, 1979a).

Van Diggelen *et al.* (1977a) showed that A9 cells deliberately infected with *M. hyorhinis* did not incorporate exogenous tritiated thymidine from the medium at all. Uninfected controls clearly displayed nuclear labeling. These authors suggested that high levels of mycoplasmal nucleoside phosphorylase were responsible for apparent lack of label incorporation. More recently, Kaplan *et al.* (1984) used [^{3}H]thymidine incorporation of cell culture supernatants as an indirect method to detect MI. This technique is essentially the same as that published by Randall *et al.* (1965). Randall *et al.* used [^{14}C]thymidine. It is interesting to compare results between the two studies. As mentioned earlier, Randall *et al.* (1965) reported counts/min/10 ml supernatant of 8100 and 6350 for mycoplasma-free and 137,000 and 60,900 for mycoplasma-infected cultures. If the cpm data reported by Kaplan *et al.* are expressed as cpm/10 ml, the results are 10,400 for mycoplasma-free cultures (avg. of 11 tests) and 44,280 for mycoplasma-infected cultures (avg. of 13 tests). However, McIvor and Kenny (1978) and Becker and Levine (1976) noted that certain mycoplasma strains are impermeable to some nucleic acid precursors. McIvor and Kenny (1978) in a study of incorporation of free bases and nucleosides among the major serological

groups of mycoplasmas, including *A. laidlawii*, *M. hyorhinis*, and *M. arginini*, reported no nucleoside incorporation among arginine-utilizing species. They also stated that uracil would be the single most useful nucleic acid precursor for incorporation studies.

Special mention should be made of the uridine/uracil (UdR/U) method developed by Schneider *et al.* (1974a). In their paper, it was proposed that ratios of 400 or above indicated mycoplasma-free cultures, since mammalian cells incorporate UdR but not U. Mycoplasmas incorporate UdR and U. Mycoplasmas also convert UdR to U by UdR-P. Ratios between 100 and 400 were considered to require retesting. This technique has fallen into disfavor. We (McGarrity *et al.*, 1979b) showed that the procedure generated questionable results in 25.2% of 115 cell cultures. Hessling *et al.* (1980) reported that results with UdR/U were totally nonconcordant with results of DNA fluorescence, agar inoculation, and electron microscopy. These authors also reported that UdR/U results were inconsistent through serial passage of the same culture. Fowler *et al.* (1983) have reported a negative UdR/C assay of the human hepatoma line PLC/PRF/5 when concomitant DNA staining and microbiological testing were positive. *Mycoplasma orale* was isolated from this cell line.

Other authors have studied other changes in nucleic acid precursor incorporation. Todaro *et al.* (1970) labeled the mycoplasma cells, which were then pelleted by ultracentrifugation. Cell cultures infected with *M. hyorhinis* incubated with [^{3}H]UdR exhibited a radioactive peak at densities of 1.22–1.24 gm/cm^3 in linear sucrose gradients. They suggested this was a presumptive diagnosis of MI. However, Sydiskis *et al.* (1981) reported that *M. hyorhinis* had a density of 1.18 gm/cm^3. The difference in these values may be due to differences in technique. Interestingly, Sydiskis *et al.* (1981) showed that *M. hyorhinis* cosedimented with mouse mammary tumor virus.

F. Electron Microscopy

Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used to detect MI. These techniques monitor cytadsorbing strains, similar to fluorescent DNA staining. However, surface artifacts can sometimes confuse diagnosis. Although it is not possible to be certain that a culture is free of mycoplasma with TEM, MI can be reasonably ascertained if characteristic mycoplasmas are observed. Techniques are described by Phillips (1978).

SEM is more cost effective and efficient than TEM. Brown *et al.* (1974) and Phillips (1978) have described methods for SEM. It can be useful to screen small numbers of monolayer cultures. We have reported difficulties in SEM of lymphoblastoid cultures (McGarrity *et al.*, 1980a). Larger numbers of cultures cannot be assayed by SEM. Phillips (1978) stated that approximately a dozen samples could be prepared for SEM in 2–3 hr, and that 100 cells could be scanned in 1 hr. Infected cultures are easily recognized, but negative cultures (at least 85% of the total) would require an hour.

IV. METHODS OF ELIMINATION

This laboratory has 54 references in its files on methods to eliminate mycoplasmas from cell cultures. This number tells us something. It implies that many of the methods are unreliable. It also implies that certain techniques may apply to some, but not all, mycoplasma species and strains. Three questions that cell biologists must ask themselves regarding elimination of mycoplasmas from cell cultures are:

1. Is the method selected reliable and efficient?
2. Will the cell culture change as a consequence of the cure process?
3. Are the time and effort invested worth it?

Whenever possible, it is much easier to discard the infected cell culture and replenish it with a stock of mycoplasma-free cell cultures. This saves time, and it also reduces the possibility that the infected culture can serve as a focus for secondary infections. It is recognized that there are occasions when suitable replacements simply are not available. It must be kept in mind, however, that the regimen used to eliminate the mycoplasma will be detrimental to the cell culture as well as to the organisms. Many procedures designed to eliminate mycoplasma essentially clone the cell culture. About 10–12 population doublings of the cultures are required to obtain confluent growth in a 25-cm^2 flask. If this occurs in a cell culture with a limited life-span, a significant portion of the life-span will be utilized through this expansion.

Various techniques have been proposed. Generally these fall into several main areas: use of antimycoplasma antisera, antibiotic treatment, passage of mycoplasma-infected tumor cell lines in nude mice, use of mouse macrophages, and agents selectively toxic to mycoplasmas. Combinations have also been used.

Use of antisera has generally been ineffective in elimination of mycoplasmas. This is due probably to the number of mycoplasmas present in infected cultures and to large surface areas where mycoplasmas can be inaccessible to antisera.

Antibiotic treatment has been successful in some reports and in some laboratories. However, the efficiency of this method varies with mycoplasma strains and antibiotic regimens. The profusion of papers in this area indicates that a single magic bullet has not been developed. Gentamicin, tylosine, neomycin, kanamycin, and related antibiotics have been the most frequently used.

Van Diggelen *et al.* (1977b) used passage in nude mice to eliminate mycoplasmas from tumor cell lines. These workers reported success in elimination

of *M. hyorhinis*. The longer the infected cell culture remains in the mouse, the greater the success rate. Fourteen to 17 days are considered minimal to enable a successful humoral antibody response against the mycoplasmas, if this is the responsible mechanism. Treatments of less than 14 days produce varying results. Schimmelpfeng *et al.* (1980) showed that freshly harvested mouse macrophages eliminated cell culture mycoplasmas.

A number of laboratories have reported the successful elimination of cell culture mycoplasmas using the technique originally published by Marcus *et al.* (1980). This method involves the selective uptake of 5-bromouracil by mycoplasmas but not by mammlian cells. Subsequent treatment with Hoechst 33258 and white light induces chromatid breaks in mycoplasma DNA. Four or more daily treatments and plating in low densities has proven successful in some laboratories. Fowler *et al.* (1983) showed that this treatment eliminated *M. orale* from human hepatoma lines. We have used it successfully in elimination of mycoplasmas from four different infected lines. Mycoplasma species were *M. orale* and *M. hyorhinis*.

V. METHODS OF PREVENTION

The ultimate sources of MI of cell cultures are bovine serum and laboratory personnel. However, it is our belief that these account for only a small percentage of infection directly. Mycoplasma-infected cultures are themselves the major source of infection. This is due to three factors: (1) high mycoplasma concentrations in cell cultures, 10^7–10^8 CFU/ml supernatant medium; (2) the ease of droplet generation when handling cell cultures; and, (3) the resistance of mycoplasmas to drying.

These three factors render infected cultures the major cause of cell culture infection. Infected cell cultures account for more MI than bovine sera and laboratory personnel! This can be implied by the low mycoplasma concentrations in contaminated sera and oral cavities. Results of environmental sampling before, during, and after trypsinization and passage of mouse 3T6 cells infected with *A. laidlawii* have been published (McGarrity, 1976). Heavy contamination of the environment occurred when infected cultures were handled and passaged.

Mycoplasmal droplets are generated in relatively large sizes during cell culture procedures. These do not remain airborne, but sediment within seconds to contaminate hands, supplies, work surfaces, and, indirectly, cell cultures. Mycoplasmas were viable for 7 days after inoculation of 0.1 ml of infected cell culture supernatants onto work surfaces. This demonstrates the urgency of effective disinfection of work surfaces between work with individual cell cultures. Disinfection of work surfaces is one of the most important procedures to prevent MI of cell cultures.

We have published guidelines that form the basis of an efficient quality control program for prevention, detection, and control of MI of cell cultures (McGarrity

et al., 1984b). These may have to be individualized or supplemented in specific laboratories.

1. Cell cultures should be acquired from reputable cell repositories such as the Institute for Medical Research, Copewood Street, Camden, NJ 08103, or the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. Catalogs are available.
2. Upon receipt in the laboratory, new cultures should be quarantined until completion of sterility, mycoplasma, and other characterization tests.
3. Carefully selected and properly standardized and controlled detection methods must be performed to assay for MI. Microbiological culture and DNA fluorescent staining using indicator cells are used in this laboratory. Cell cultures should be assayed at least quarterly, more frequently if there is a history of mycoplasmal infection or if a large number of cultures from outside laboratories are handled.
4. The mycoplasma-free cell culture should be preserved in liquid nitrogen when it is first shown to be free of mycoplasmas and other adventitious agents.
5. Antibiotics should not be used in routine carriage of cell cultures. Antibiotics are useful in preparation of primary cell cultures and for selected single experiments that have an increased potential for infection. Stock cultures, however, should always be maintained in antibiotic-free media to prevent undetected infection.
6. Work surfaces should be carefully disinfected after work with individual cell cultures has been completed. Disinfectant solutions should be discarded regularly to prevent inactivation of the disinfectant and microbial overgrowth.
7. Prohibition of mouth pipetting.
8. Media, especially the serum, components should be tested for sterility before they are used in cell culture media. Serum should be assayed for mycoplasmas by the large volume test procedure of Barile and Kern (1971). Serum should also be pretested for growth promotion using cell cultures appropriate for each laboratory.
9. Discarded glass and plastic wares and spent media should be carefully disinfected.
10. Disposal of cell culture materials in a central sterilization area.
11. Use of certified laminar flow biological safety cabinets using leakfree high-efficiency particulate air (HEPA) filters. HEPA filters have a *minimum* efficiency of 99.97% at 0.3 μm; the efficiency actually increases for particles smaller than 0.3 μm. Horizontal flow laminar air flow cabinets should not be used when handling cell cultures, microorganisms, or chemicals that may effect humans.
12. Effective housekeeping procedures to minimize contamination by environmental sources. Particular attention should be paid to chemical disinfection of floors, sinks, and faucets.

13. The prompt autoclaving of cell cultures that are found to contain mycoplasmas. If attempts to cure the infection will be made, the culture should be quarantined.

14. Careful aseptic techniques.

15. Periodic review and updating of laboratory procedures. Written protocols for all laboratory procedures should be available to all personnel and followed carefully. New equipment and procedures should be analyzed to determine if they can contribute to cell culture variability.

16. Laboratory technicians are the most effective monitor of unexpected changes in cell cultures. Effective communication between laboratory technicians and supervisory personnel is essential.

These procedures are prudent and nonrestrictive. We have likened MI to venereal disease of cell cultures (McGarrity *et al.*, 1984a). Cell biologists must recognize that the difficulties associated with cell culture mycoplasmas are preventable if effective and simple quality control practices are designed and enforced.

ACKNOWLEDGMENTS

The authors acknowledge and appreciate the many technical contributions of Judi Sarama, Veronica Vanaman, and Theresa Schuck. We thank Riley Hanson for artwork. Some of our studies have been supported by Grant AI 15748-04 and by Contracts #NO1-GM-4-2100 and #NO1-AG-0-2100.

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11 / LABORATORY DIAGNOSIS OF MYCOPLASMA INFECTIONS

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I. Introduction 391
II. Pathogenic Associations 392
III. Specimens for Study 393
A. Collection 393
B. Transport 394
C. Preparation 394
D. Storage 394
IV. Culture Systems 395
A. Quality Control 395
B. Selective Media 396
C. Recognition of Growth 396
D. Speciation of Isolates 397
V. Antigen Detection Methods 398
A. Direct Immunofluorescence 398
B. Enzyme-Linked Immunosorbent Assay 399
C. Radioimmunoassay 399
VI. Serodiagnosis 400
VII. Future Prospects and Developing Methodologies 400
References 402

I. INTRODUCTION

In "The Mycoplasmas," Volumes I–III, no specific attention was given to the subject of laboratory diagnostic studies for mycoplasma infections. This chapter is designed to deal with the general principles which are involved in diagnosis based on organism replication, antigen detection, and serology. Since the mycoplasmas are a highly heterogeneous group, details suitable for diagnosis of

THE MYCOPLASMAS, VOL. IV

APPENDIX 3. RELATED PROCEEDINGS

There are no related proceedings.

APPENDIX 4. JURISDICTIONAL STATEMENT

This brief is filed in support of the appeal of the Final Rejection mailed October 6, 2008. The appeal is authorized by 35 U.S.C. 134(a) and is filed pursuant to the Notice of Appeal filed January 6, 2009. The appeal is being filed on, or before March 6, 2009 and is thus timely.